CHARACTERIZATION OF MICRORNA DEREGULATION IN HEPATOCELLULAR CARCINOMA

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CHARACTERIZATION OF MICRORNA DEREGULATION IN HEPATOCELLULAR CARCINOMA

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SUMMARY

Hepatocellular carcinoma (HCC) is the 5th most common cancer worldwide with an annual global mortality of ~700,000. HCC is a very complex disease and the role of miRNAs in HCC is not fully understood. In this study, we profiled miRNA expression in tumor versus paired adjacent non-tumor tissues from 100 HCC patients and identified a miRNA signature of 66 significantly (FDR<0.000) differentially expressed miRNAs associated with HCC. We subsequently characterized let-7a down-regulation and miR-224 up-regulation. We demonstrated that hepatitis B virus X protein (HBx) down-regulates let-7a and let-7a negatively regulates cell proliferation through targeting Signal Transduction and Activator of Transcription 3 (STAT3). The HBx-let-7-STAT3 pathway supports cell proliferation in HBx-expressing cells. Additionally, we demonstrated that over-expression of miR-224 increases cell proliferation by targeting SMAD family member 4 (SMAD4) and sensitizes cells to apoptotic cell death by targeting Apoptotis Inhibitor 5 (API5). We observed statistically significant ($p<0.05$) inverse correlation between let-7a & STAT3, miR-224 & SMAD4 and miR-224 & API5 in HCC patients, demonstrating the clinical relevance of our observation. Therefore, our work contributed to the identification of miRNA deregulation in HCC and the understanding of the functional relevance of two such miRNA deregulations with respect to HCC.

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LIST OF ABBREVIATIONS

3'UTR	3' untranslated region
AFP	Alpha-fetoprotein
API5	Apoptosis inhibitor 5
B-gal	Beta-galactosidase
bp	Base pairs
ChIP	Chromatin immunoprecipitation
CT	Threshold cycle or Computed Tomography
Cy3/Cy5	Cyanine 3 / Cyanine 5
DMEM	Dulbecco's modified Eagle's media
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDR	False Discovery Rate
GO	Gene Ontology
HBV	Hepatitis B virus
HBx	Hepatitis B virus X protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
JAK	Janus family tyrosine kinase
LMW	Low molecular weight
LOWESS	Locally weighted scatterplot smoothing
MAPK	Mitogen-activated protein kinase
miR/miRNA	microRNA
miRISC	miRNA induced silencing complex
mRNA	Messenger RNA
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
ORF	Open reading frame
PCR	Polymerase chain reaction
PDCD4	Programmed cell death 4
PEI	Percutaneous alcohol injection
PIAS	Protein inhibitors of activated STAT3
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary transcript miRNA
PTEN	Phosphatase and tensin homolog
PP2A	Protein phosphatase 2A
RAS	Rat sarcoma viral oncogene homolog

RFA	Radiofrequency ablation
RNA	Ribonucleic acid
rpm	Revolutions per second
RT-qPCR	Reverse transcription quantitative PCR
SAM	Significance Analysis of Microarrays
SMAD4	SMAD family member 4
SOCS	Suppressor of cytokine signaling proteins
STAT3	Signal transducer and activator of transcription 3
TACE	Transarterial chemoembolization
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Hepatocellular carcinoma

1.1.1 HCC epidemiology

Hepatocellular carcinoma (HCC) is the primary cancer of the liver which is the fifth most common cancer among men worldwide and eighth among women (Figure 1.1.1) [1]. The distribution of HCC shows significant geographical variation. More than 80% of HCC cases occur in Asia and sub-Saharan Africa while its incidence is relatively low but steadily rising in Japan, UK, Germany and USA over the past two decades (Figure 1.1.2) [2]. HCC is almost always lethal, with less than six months of survival time upon diagnosis and a median five year survival rate of 5-9 %. With more than 700,000 deaths annually, HCC is ranked the third leading cause of cancer-related deaths worldwide [3], posing a serious health and economic burden to the affected individuals and the countries.

1.1.2 HCC etiology

HCC is a very heterogeneous disease with many etiologic factors. Table 1.1.2 summarizes the many viral and environmental factors associated with HCC. Chronic hepatitis viral infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) is the dominant risk factor for HCC carcinogenesis. The impact of hepatitis virus infection on HCC varies greatly from region to region. HBV is implicated in approximately 80 % of HCC cases in Asia and sub-Saharan Africa where HBV infection is endemic and 10-15 % of the general population are chronic HBV carriers [4]. In contrast, HCV

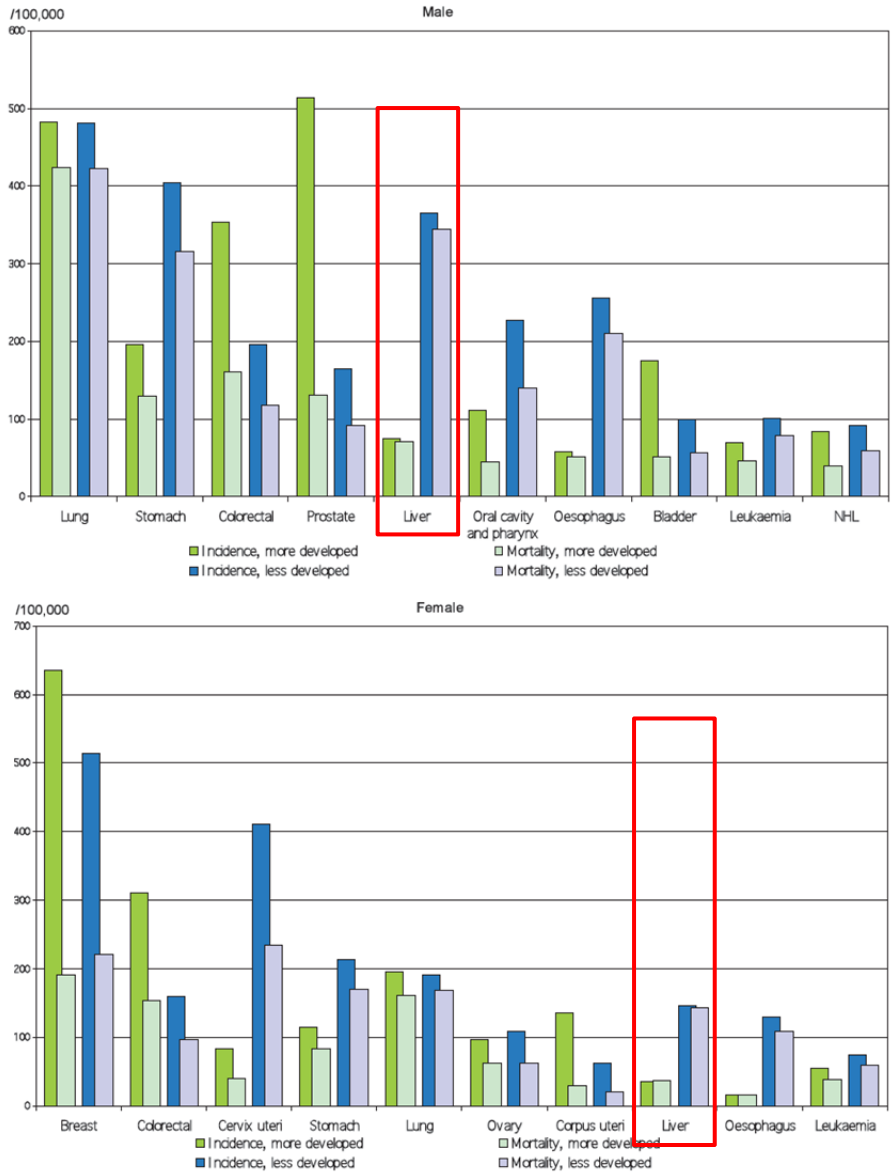

Figure 1.1.1 The incidence and mortality of the top ten most common cancers worldwide. Cancer incidence and mortality in more-developed and less-developed countries are plotted as bar charts and ranked according to overall incidence in males (top) and females (bottom) population. The liver cancer incidence in males and females are boxed in red.
Sources: World Cancer Report 2008.

infection, which infects 2-3 million people each year, is particularly implicated in HCC in the more developed countries such as Japan and the USA. Worldwide, the proportion of HCC attributable to chronic hepatitis is about 55 % for HBV and 30 % for HCV [3]. This is conservative estimate, as it only accounts for cases with detectable HBV surface antigen (HBsAg) in the blood stream and excludes cases with occult HBV infection, where there is persistent viral DNA with no release of HBsAg in the blood stream. Therefore, the overall contribution of hepatitis viral infection to HCC may be close to 90 %.

1.1.3 HBV and HCC

There are an estimated 400 million chronic HBV carriers worldwide, making HBV the single most important causal factor of HCC.

HBV has a relaxed circular, partially double-stranded DNA genome of approximately 3.2 kilobase pairs (bp) in size (Figure 1.1.3). The HBV genome comprises four overlapping open reading frames (ORFs) in the minus DNA strand of the virus. These ORFs encode viral peptides including: the viral envelope (pre-S/S), the viral nucleocapsid or core (pre-core/core), viral polymerase and the X protein (HBx) [5].

The mechanism of HBV in contributing to HCC is still not fully understood. Our current knowledge suggests that at least three overlapping mechanisms may be involved in HBV-associated HCC. First, persistent HBV infection induces oxidative stress damage in hepatocytes, resulting in significant cell death and compensatory cell proliferation. This creates a deregulated and inflammatory environment in which the transformed cells will gain survival advantage due to their increased capacity to proliferate and

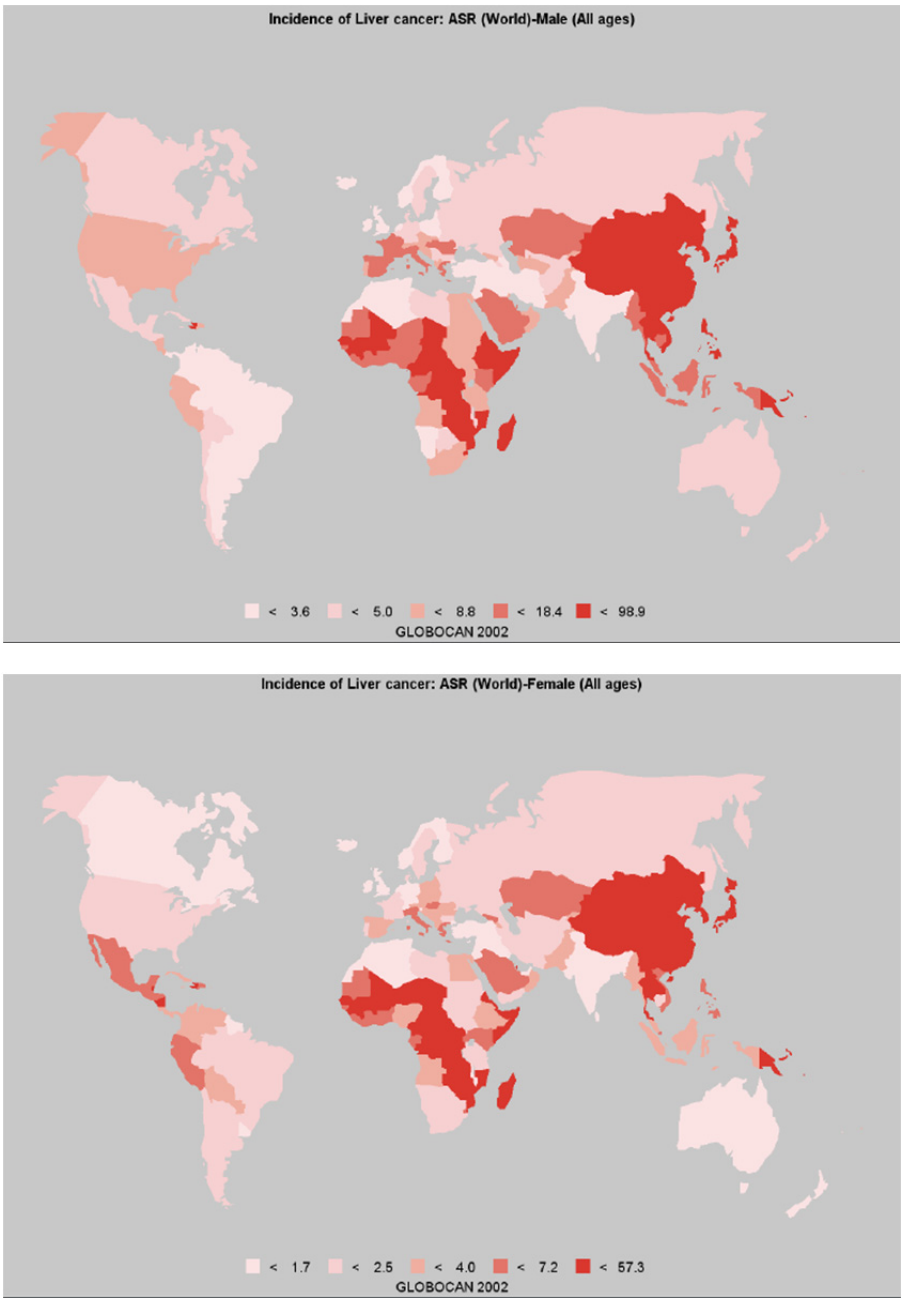

Figure 1.1.2 Incidence of liver cancer worldwide 2002. HBV is particularly prevalent in Asia and sub-Saharan Africa, as highlighted in intense red color. Unit of measurement: 100,000. Sources: World Cancer Report 2008.

Table 1.1.2 Major etiologic factors associated with HCC.

Major Etiologic Factors
Hepatitis B virus infection
Hepatitis C virus infection
Alcohol consumption
Aflatoxin B1
Tobacco smoking
Obesity/diabetes/fatty liver/ iron overload

decreased sensitivity to apoptosis signals, favoring tumorigenesis. Second, even though HBV does not contain an integrase gene, the integration of HBV genome into host genome is still very frequently observed in HCC patients who are chronic HBV carriers. Such integration events create opportunities for insertional mutagenesis to occur and this can potentially activate cellular proto-oncogenes to oncogenes or inactivate cellular tumor suppressor genes, in the vicinity of the integration point. Previous small scale studies have not identified any consensus HBV integration sites in the human genome. Third, the expression of HBV encoded proteins such as HBx has a significant impact on the host cellular gene expression and pathways. The HBx gene is the most common viral open reading frame integrated into the genome of HCC patients and the HBx protein is often found to be selectively over-expressed in the tumors of HCC [6-8]. HBx lacks a DNA binding domain and is widely believed to function as a promiscuous trans-co-activator by interacting with various cellular factors to deregulate many host genes such as tumor suppressor p53 (TP53) and v-myc myelocytomatosis viral oncogene homolog (avian) (c-Myc). HBx is also reported to activate a number of cellular signaling pathways such as mitogen-activated protein kinase (MAPK), Janus family tyrosine kinase (JAK)/signal transducer and activators of transcription (STAT) pathways [9, 10]. Hence, HBV contributes to HCC oncogenesis by impacting on multiple crucial cellular processes through a combination of highly complex mechanisms, resulting in the long term destabilization of normal hepatocyte function and a favorable environment for HCC carcinogenesis.

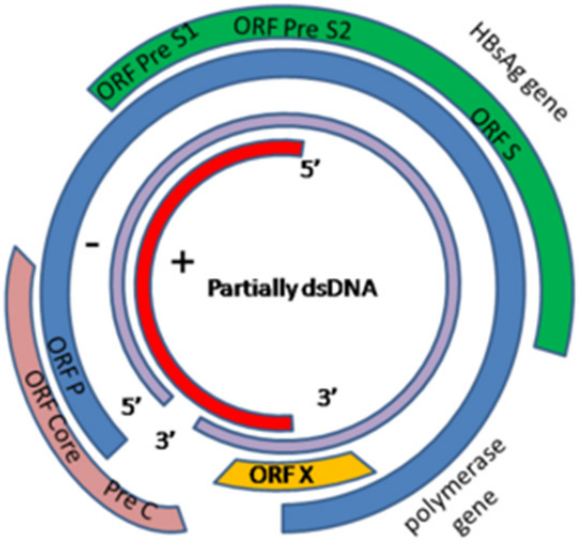

Figure 1.1.3 Hepatitis B virus genome organization. The HBV genome is a partially double-stranded DNA with four overlapping open reading frames, Polymerase (P), Surface (S), Core (C) and X gene (X). (Source: http://en.wikipedia.org/wiki/File:HBV_genome.png, free license from Wikimedia Commons.)

1.1.4 HCC prevention, detection and treatment

Since 1980s, safe and affordable HBV vaccine has been made available through recombinant technology. Mass vaccine programs have been shown to be effective at reducing the incidence rate of acute HBV infection and chronic HBV carriers, with a corresponding drop in HCC incidence in the vaccinated population [11]. As a result, World Health Organization (WHO) has recommended national HBV immunization programs for all countries in 1992 and by 2002 more than 135 countries have included HBV vaccine in their national vaccination programs. Unfortunately, no equivalent vaccine is available for HCV. Reduction of risky behaviors and the improvement of hygiene standards are currently the main measures for prevention of HCV.

Early detection of HCC has been difficult as the progression of HCC is largely asymptomatic. Currently, surveillance of HCC relies mainly on monitoring serial α-fetoprotein (AFP) levels and regular ultrasound or computed tomography (CT) examination [12]. A definitive diagnosis of HCC depends on histological analysis via fine needle biopsy. However, elevated AFP as a biomarker lacks specificity and the ultra-sound or CT scan is too costly for a general screening application. A simple plasma-based test will be an important contribution to HCC screening programs.

The only treatment modalities that consistently convey survival advantage to HCC patients are surgical resection and liver transplantation. Surgical resection is to remove a tumor together with surrounding liver tissue while preserving enough liver remnants for normal body function. This treatment offers the best prognosis for long-term survival, but unfortunately

only 10-15% of patients are suitable for surgical resection. This is often due to late stage presentation of HCC tumor at the time of diagnosis and associated poor liver function of the patients [13]. Resection in cirrhotic patients carries high morbidity and mortality. For better prognosis after resection, the expected liver remnant should be more than 25% of the total size for a non-cirrhotic liver and more than 40% of the total size for a cirrhotic liver. The overall recurrence rate after resection is 50-60% [14, 15]. On the other hand, liver transplantation is used as a curative therapeutic approach. However, this approach is very much limited by the low survival rate of patients post operation due to rejection and shortage of appropriate liver donors. Non-surgical treatment of HCC such as chemotherapy, hormone therapy and radiotherapy has been largely disappointing. Regional therapies such as percutaneous alcohol injection (PEI) [16], radiofrequency ablation (RFA) [17] and transarterial chemoembolization (TACE) [18] are associated with too many side effects and doubtful long term benefits for HCC patients. They are mainly used as options for pre-resection treatment or prolonging survival time for liver transplantation.

The current limitation on the availability of easy detection and effective treatment measures highlights the urgent need to better understand HCC as a complex disease in the hope that novel targets for early detection and better treatment can be identified. Recent advances in microRNA (miRNA) research has presented this group of small non-coding RNAs as promising new targets where novel therapeutic and diagnostic strategies can be designed to tackle human cancers. In this thesis, we will focus on the role of miRNAs in HCC.

1.2 microRNA and cancer

MiRNAs are a class of small non-coding RNAs of ~ 22 nucleotides in mature sequences and negatively regulate gene expression at the post-transcriptional and/or translational level. They were first discovered by Ambros and colleagues in 1993 [19] when they studied the larval development in *C. elegans*. Since then miRNAs have been shown to be abundantly expressed in viruses [20], plants [21] and animals [22]. According to the miRBase Release 15, there are a total of 14,197 miRNAs identified to date, out of which, 940 miRNAs are found in humans [23-25]. Many miRNAs show sequence and function conservation between distantly related organisms, suggesting that this class of small RNAs is an integral part of essential cellular processes [26]. For example, *Lethal-7* (*let-7*) was discovered to be responsible for the developmental transition of L4 larvae to the adult cell fates [27] in *C. elegans* and later found to be evolutionarily conserved to regulate development in *Drosophila*, zebrafish, annelids, mollusks [26] and mouse [28]. The human let-7 family contains 12 homologue members. Despite their recent discovery, microRNAs represent a novel class of gene regulators that may have an ancient origin. Their discovery has opened up a new dimension in our understanding of gene regulation.

1.2.1 miRNA biogenesis

As illustrated by Figure 1.2.1, microRNAs are encoded in the genome and transcribed by RNA polymerase II as primary transcripts that are called pri-miRNAs. Pri-miRNAs are typically 3 to 4 kilobase long single-stranded RNAs with 5' cap, 3' poly(A) tail and profound secondary structure [29, 30]. They

can contain one or more precursor miRNA (Pre-miR) sequences which are processed in the nucleus by microprocessor complex comprising of nuclear RNase III, Drosha, and the double-stranded RNA binding protein, Pasha/DGCR8, into ~70 nuceotide pre-miRNAs [29-31]. Pre-miRNAs are then actively exported to the cytoplasm through exportin-5 in association with RAN-GTPase [32, 33]. In the cytoplasm, another RNase III, Dicer, further processes the pre-miRNAs into ~ 22 nucleotide miR: miR* duplex [34, 35] of which one of the strands (miR strand) is incorporated into the multi-protein RNA induced silencing complex (miRISC) while the other strand (miR* strand) is degraded. This strand preference is probably due to the difference in the thermodynamic stability of the strands [36]. In mammalian system, the functional miRISC carrying the mature miR can bind to the 3'untranslated region (3'UTR) of its target gene mRNA to result in either mRNA degradation (for nearly perfect complementary base-paring) or protein translation inhibition (for imperfect complementary base-paring). The mechanism of inhibition will depend on the miR sequence, the target mRNA sequence and the exact composition of the miRISC protein complex [37, 38].

1.2.2 miRNA and cancer

The importance of microRNAs to cancer is highlighted by the observation that ~50% of miRNA genes are located in cancer associated genomic regions or fragile sites [39, 40], which are frequently amplified or deleted in tumorigenesis. Global repression of microRNA processing machinery (Drosha, Pasha/DGCR8 and Dicer1) promotes cellular transformation and miRNA processing-impaired cells formed tumors with accelerated kinetics in mouse model, implicating the role of mature miRNAs

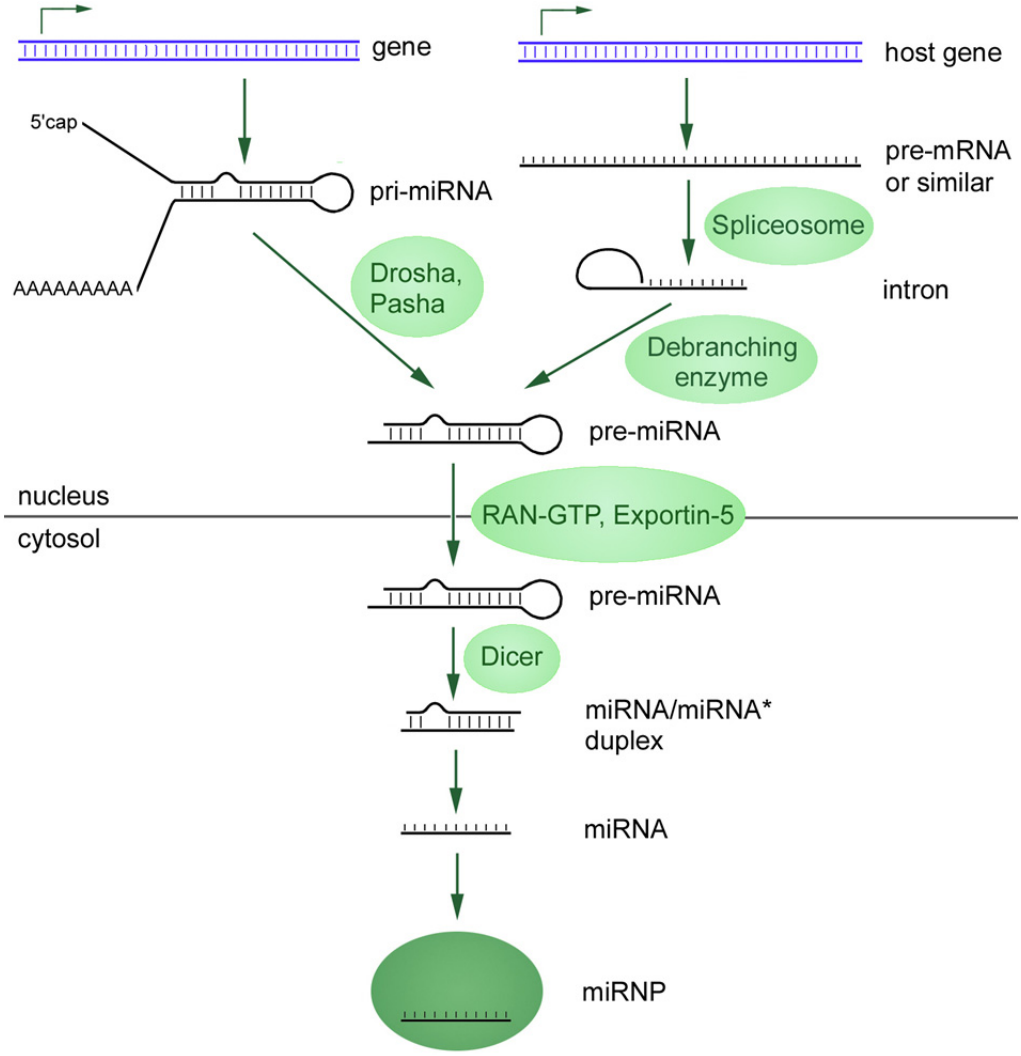

Figure 1.2.1 micoRNA biogenesis. miRNAs are transcribed from their genomic loci as long primary transcript miRNA, Pri-miRNAs, which are processed to hairpin-looped precursor miRNAs, Pre-miRNAs, in the nucleus. Pre-miRNAs are then actively transported into the cytoplasm where they will be processed by Dicer to generate mature miRNAs to be loaded into the multi-protein miRNA induced silencing complex (miRISC).
Source: http://en.wikipedia.org/wiki/File:MiRNA-biogenesis.jpg#filehistory, licensed under Creative Commons Attribution-ShareAlike 3.0 License.

in cancer-related processes [41]. Large-scale microRNA expression profiling of human cancers have revealed that miRNA deregulation is frequently associated with many cancer types originating from the blood [42-47], brain [48-50], thyroid [51-53], breast [54], lung [55-57], tongue [58], nose and pharynx [59], liver [60-63], the gastro-intestinal system (esophageal cancer [64], gastric cancer [65], pancreatic cancer [66, 67] and colorectal cancer [68, 69]) and genitor-urinary system (cervical cancer[70], ovarian cancer [71, 72] and prostate cancer[73, 74]).

Table 1.2.2 summarizes our current knowledge on miRNA expression profiles in various human cancers. In these studies miRNA expression in tumors is compared against the paired non-tumorous tissues from cancer patients and significantly up- and down-regulated miRNAs are indicated with red/up-arrows and green/down-arrows respectively. More than one quarter of known human miRNAs (175 out of 678 miRNAs) have been reported to be de-regulated in at least one cancer type. This is only a gross underestimation as the majority of the known miRNAs are only identified in recent years and not included in previous miRNA expression profiling studies. This places microRNAs as the largest class of gene regulators implicated in cancer-related processes and of which we still know very little about. Table 1.2.2 has also revealed some interesting patterns of miRNA expression in cancers. Of these cancer-implicated miRNAs, miR-21 is the most commonly up-regulated miRNA in both solid and hematological tumors, consistent with the report in 2006 by Volinia et al [74]. In addition, miR-155, miR-181b, miR-221 and miR-222 are also frequently up-regulated in cancers of the blood, brain, thyroid and the gastro-intestinal (GI) systems, and to a lesser extent in liver

Table 1.2.2 miRNAs that are differentially expressed in human cancers and their validated *in vivo* targets.

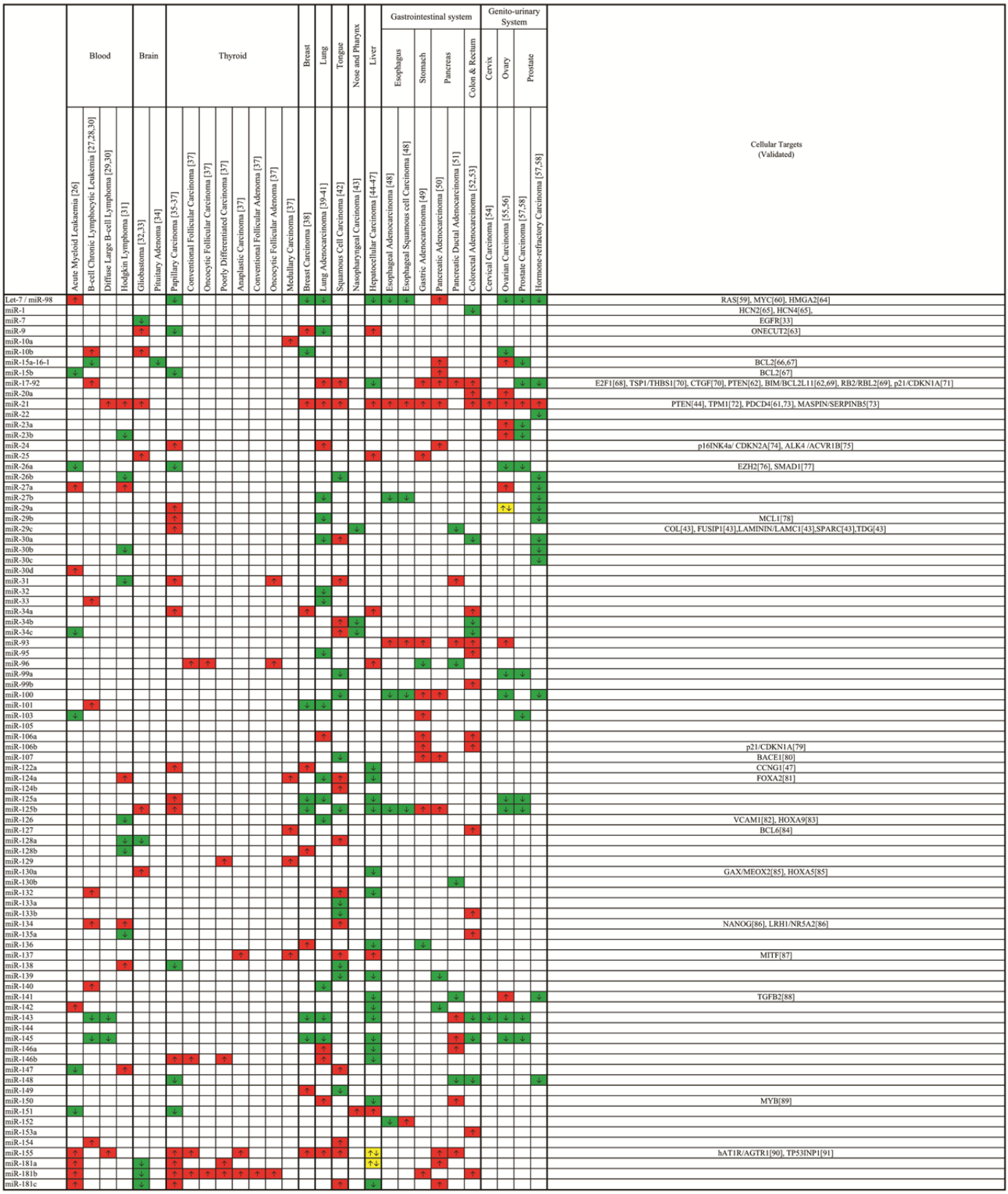

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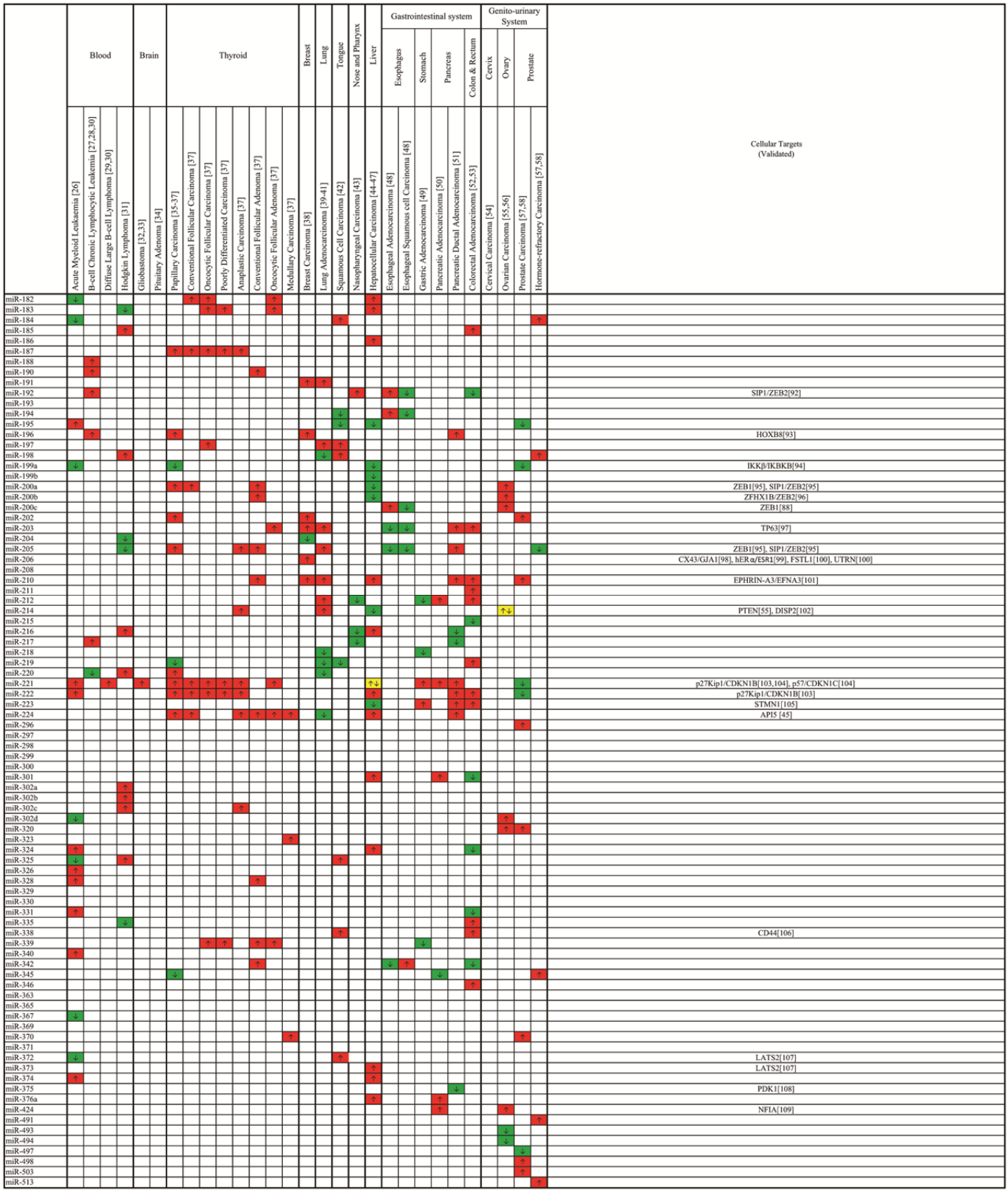
Table 1.2.2 continued: miRNAs that are differentially expressed in human cancers and their validated in vivo targets.



(Published data)

cancer, lung cancer and breast cancer. In contrast, the let-7/miR-98 cluster is commonly down-regulated in tumors of the thyroid, breast, lung, upper GI and the genitor-urinary system. Similarly miR-143 and miR-145 are frequently down-regulated in the hematological tumors and solid tumors of the breast, lung, prostate and the lower GI system. Such common deregulation of miR expressions across various tumor types suggests that these miRNAs may be involved in crucial cellular pathways that are commonly deregulated in cancer development. Indeed, functional studies have demonstrated that let-7/miR-98 negatively regulates Rat sarcoma viral oncogene homolog (RAS) [75] and v-myc myelocytomatosis viral oncogene homolog (avian) (MYC) [76] whilst miR-21 negatively regulates Phosphatase and tensin homolog (PTEN) [60] and Programmed cell death 4 (PDCD4) [77], which are important cellular proto-oncogenes or tumor suppressors that regulate cell growth, cell proliferation and apoptosis. The deregulation of these important cellular processes represents an important hallmark of cancer. Other than the common deregulation of miRNAs, we can also identify some tumor type specific pattern of miR expression in Table 1 as well. The miR-17-92 cluster and miR-93 are frequently reported to be up-regulated mainly in cancers of the GI system. A higher proportion of miRNAs were reported to be up-regulated in thyroid tumors whilst a greater percentage of miRNAs were reported to be down-regulated in nasopharyngeal cancer and cancers of the genito-urinary system. This suggests that some tumor-specific mechanisms may be in place to favor a specific miR profiles depending on the tumor micro-environment. It is also worth noting that miR-105, miR-144, miR-193 and miR-199b are seldom reported to be deregulated in cancer, despite their relatively early

Table 1.2.3.(1) Recent publications on systematic profiling of microRNA deregulation in human HCC.

Author	Year	Journal	Origin	Sample size	Comparison	No. of mature miRNAs examined	Profiling method	miRNA studied	Functional study	Target identified
Murakami *et al.*	2006	Oncogene	Japan	25	25T vs 25 Adj NT	180	Microarray	-	-	-
Gramantieri *et al.*	2007	Cancer Res.	Italy	17	17 T vs 21 LC	238	Microarray	miR-122a	-	CCNG1
Meng *et al.*	2007	Gastro-enterology	USA	3	3 T vs 3 N.L	-	Microarray	miR-21	Proliferation, Migration and invasion	PTEN
Huang *et al.*	2008	J Gastroenterol Hepatol	China	10	10 T vs 10 Adj. NT	331	Microarray	-	-	-
Wang *et al.*	2008	J Biol Chem	Singapore	19	19 T vs 19 Adj. NT	182	qPCR	miR-224	Apoptosis, proliferation	API5
Jiang *et al.*	2008	Clin Cancer Res.	USA	54	54 T vs 54 adj. NT	196	qPCR	-	-	-
Varnholt *et al.*	2008	Hepatology	Germany	52	52 T vs 3 N.L	80	qPCR	-	-	-
Ladeiro *et al.*	2008	Hepatology	France	55	55 T vs 30 NT	250	qPCR	-	-	-
Connolly *et al.*	2008	Am J Pathol.	China	19	19 T vs 19 Adj. NT	-	MiRNA cloning	miR-17-92, miR-21	Apoptosis, transformation, cell proliferation	-
Su *et al.*	2009	Cancer Res.	China	20	20 T vs 20 Adj. NT	308	Microarray	miR-101	Colony formation, tumorigenicity, apoptosis	MCL1
Huang *et al.*	2009	Hepatol Res.	China	20	20 T vs 20 Adj. NT	114	Bead-based flow	miR-338	-	-
Ura *et al.*	2009	Hepatology	Japan	26	26 T vs 9 N.L.	188	qPCR	-	-	-
Pineau *et al.*	2010	PNAS	France	104	104 T vs 21 N.L.	215	Microarray	miR-221	Cell growth, tumorigenicity	DDIT4
Wong *et al.*	2010	Clin Cancer Res.	China	20	20 T vs 12 L.C	156	qPCR	miR-222	Migration, invasion	PPP2R2A

Sample size is the number of the HCC tumor samples used in the initial miRNA profiling. Comparison is the design of experiments, HCC tumor samples (T) vs paired adjacent non-tumor samples (Adj. NT) or cirrhotic liver samples (LC) or normal liver samples (NL)

discovery, suggesting that these miRNAs probably take part in cellular house-keeping processes that have little role in oncogenesis.

Although much is known about the aberrant miR expression pattern associated with various cancers, much less is known about the functional relevance of such miR deregulation or the in vivo miR targets. Table 1.2.2 also summarizes a total of 65 non-overlapping experimentally validated miR direct cellular targets reported to date [49, 59-61, 63, 71, 75-124]. Functional annotation with Gene Ontology (GO) and KEGG pathways reveals that these 65 validated miRNA target genes show a significant enrichment in the classical cancer-associated pathways such as transcription, cell-cell adhesion and signaling, cell cycle regulation, cell proliferation and apoptosis, strongly implying that the deregulation of these miRNA target genes may play significant roles in carcinogenesis. However, as predicted by miR target prediction algorithms (miRanada[24], PicTar[125] and TargetScan[126]), each individual miRNA can potentially regulate hundreds of cellular gene targets and the identification of these *in vivo* miRNA targets still represent a real challenge to the comprehensive understanding the miRNA regulated networks that impact significantly in cell differentiation, cell proliferation and apoptosis[127].

1.2.3 microRNA implication in HCC

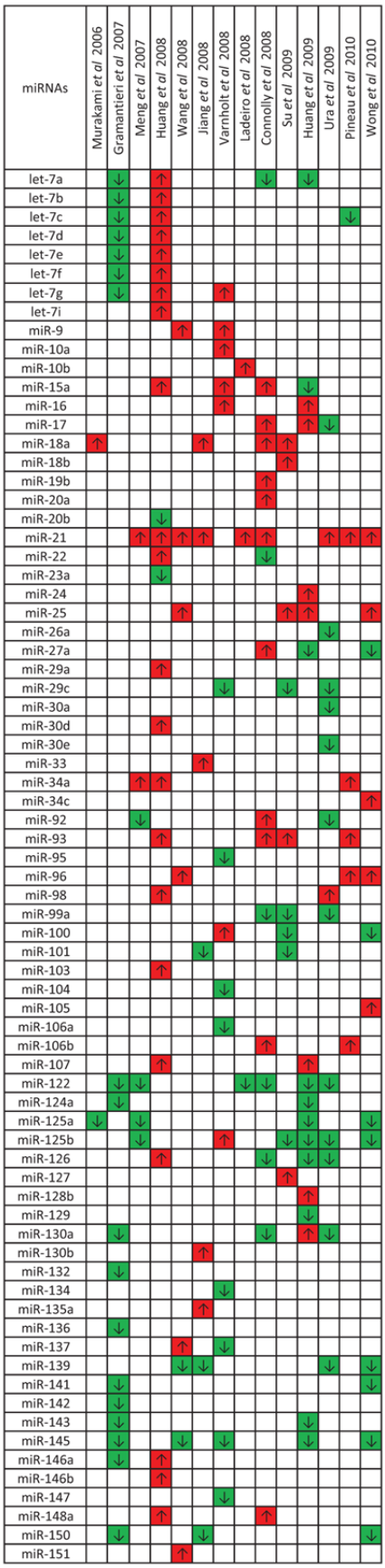
1.2.3.(1) microRNA deregulation is frequently observed in HCC

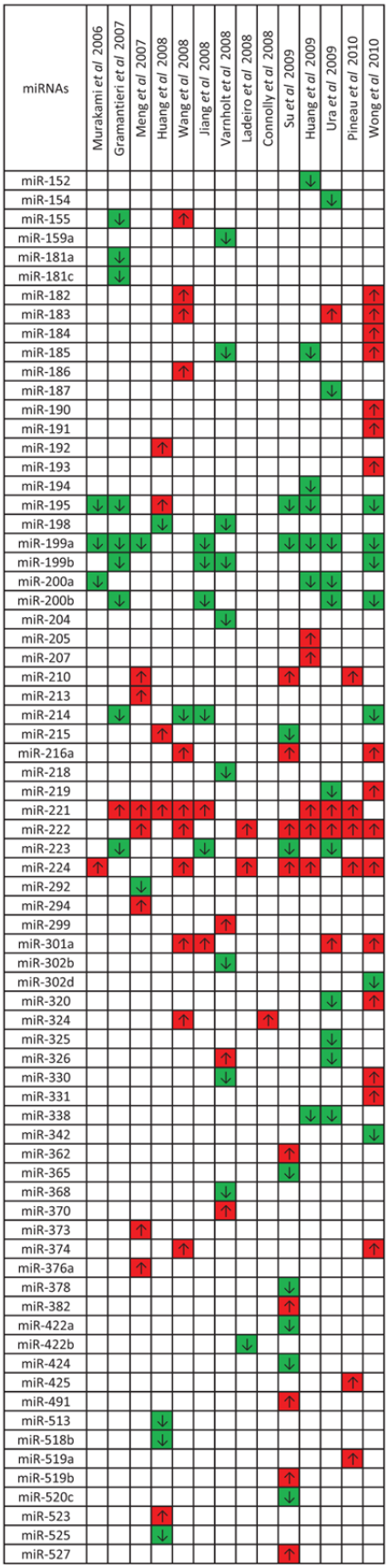
Like other cancers, aberrant microRNA expression features significantly in HCC. Since Murakami *et al.* first reported miRNA deregulation in human HCC in 2006, a total of 14 publications on comprehensive profiling of miRNA

expression in HCC have been reported in reputable journals (Table 1.2.3.(1)) [60-63, 128-137]. HCC patient samples included in these 14 studies are from diverse geographic origins with very different etiologies of HCC, such as China (5 studies), France (2 studies), Germany (1 study), Italy (1 study), Japan (2 studies), Singapore (1 study) and United States (2 studies). Expression of hundreds of miRNAs has been examined between HCC tumor samples and either paired adjacent non-tumor samples, unpaired non-tumor or cirrhotic liver samples or normal liver samples, utilizing different profiling technologies such as bead-based flow, reverse transcription quantitative PCR, miRNA cloning or microRNA microarrays.

Table 1.2.3.(2) summarizes the list of aberrantly expressed miRNAs in HCC as reported by these 14 studies. A total of 147 miRNAs have been shown to be deregulated in HCC by at least one of the 14 studies. 80 of these 147 miRNAs (~55 %) are reported by a single study where 67 of them (~45 %) have been reported to be deregulated in HCC by at least two studies. The pattern of deregulation for 41 of these 67 miRNAs (~61 %) has been consistent while discrepancies in reported deregulation exist for the remaining 26 miRNAs (~39 %). These diverse miRNA signatures may be a result of the highly heterogeneous makeup of HCC patients included in the different studies, differing greatly by their etiologic factors based on the geographical origins and ethnicity, compounded by the difference in number of miRNAs included in each study and the profiling methods used. This re-emphasizes the complexity of HCC as a disease and much effort is needed to elucidate the role of such miRNA deregulation in association with the many etiologic factors involved.

Table 1.2.3.(2) List of microRNAs deregulated in HCC.





1.2.3.(2) Deregulated miRNAs in HCC impacts cellular functions

MiRNAs which have been consistently reported to be aberrantly expressed by multiple studies, irrespective of the nature of the patient samples or the profiling technologies, are most likely to be involved in hepatocellular carcinogenesis. Table 1.2.3.(3) summarizes the 26 miRNAs which have been consistently reported by three or more studies. Virtually all of the 26 miRNAs have been reported to be deregulated in at least one other type of cancer (Review [138]), suggesting a panel of miRNAs may be needed to achieve specificity for characterizing HCC. Table 1.2.3.(3) also summarizes a total of 175 experimentally validated cellular miRNA targets for these 26 miRNAs, out of which 161 are non-overlapping targets [59-61, 63, 71, 88, 89, 109-111, 116, 118-120, 136, 137, 139-240]. Many of these miRNA targets are very crucial cellular genes such as RAS (miR-214), MYC (miR-145) and PTEN (miR-21, miR-214 and miR-221/222) whose deregulation will have detrimental effect on the cell. Functional annotation of these miRNA targets with Gene Ontology (GO) has revealed significant enrichment in cancer-implicated cellular processes such as cell proliferation, cell death and cell differentiation, in favor of tumorigenesis [241]. Hence, miRNA deregulation can play an important role in hepatocellular carcinogenesis by interfering with biological and molecular functions of the cell.

1.2.3.(3) microRNAs as prognostic and diagnostic markers for HCC

Current classification of HCC replies exclusively to clinical parameters and miRNA profile may have the potential to improve our ability to classify HCC and stratify prognosis. MiRNA expression signatures have been shown

Table 1.2.3.(3) List of microRNAs deregulated in HCC, consistently reported by at least three profiling studies

miRNAs	Murakami *et al* 2006	Gramantieri *et al* 2007	Meng *et al* 2007	Huang *et al* 2008	Wang *et al* 2008	Jiang *et al* 2008	Varnholt *et al* 2008	Ladeiro *et al* 2008	Connolly *et al* 2008	Su *et al* 2009	Huang *et al* 2009	Ura *et al* 2009	Pineau *et al* 2010	Wong *et al* 2010	Genomic location	Genomic cluster	Deregulation in cancers of other origins (5)	Validated targets (Liver)	Other validated targets
miR-18a	↑					↑			↑	↑					13q31.3	miR-17-92	Blood, colon,stomach, lung	ESR1(20)	CCN2(21)
miR-21			↑	↑	↑	↑		↑	↑			↑	↑	↑	17q23.2	-	Brain, lung, stomach, prostate, colon, ovary, cervix, breast, etc.	PTEN(8), PDCD4(23), RECK(23), RHOB(22), PELI1(25)	BTG2(24), BMPRII(27), IL-6R(27), SOCS5(27) ,CDK6(27) , TGFBR2(31), HNRNPK(33), TP63(33), MTAP(26), SOX5(26), RASGRP1(32), JAG1(30), WNT1(30), SPRY2(34), NFIB(28), TIMP3(29), SERPINB5(37), TPM1(36), NCAPG(35), RTN4(35), DERL1(35), BASP1(35), PLOD3(35), MARCKS(40), LRRFIP1(41), FASLG(45)
miR-25					↑					↑	↑			↑	7q22.1	miR-25/93/106b	Brain, stomach	BIM(42)	PTEN(44)
miR-29c							↓			↓		↓			1q32.2	miR-29b-2/29c	Thyroid, nose & pharynx, pancrease	BCL2(47), MCL1(47)	LAMC1(46), DNMT3A(38), DNMT3B(38), COL3A1(46), COL4A1(46), COL15A1(46), TDG(46), FUSIP1(46), COL1A1(46), COL1A2(46), COL4A2(46), SPARC(46), PIK3R1, CDC42(43), CDK6(48)
miR-34a			↑	↑									↑		1p36.22	-	Thyroid, breast, colon	MET(39)	CCND1(55), CDK6(55), MYCN(57), SIRT1(58), JAG1(30), WNT1(30), BCL2(56), MYC(49), NOTCH1(52), NOTCH2(52), MEK1(51), FOXP1(54)
miR-93				↑					↑	↑			↑		7q22.1	miR-25/93/106b	Esophagus, stomach, pancrease, colon, ovary	E2F1(42)	TP53INP1(59), FUS1(50), VEGF(53)
miR-96					↑								↑	↑	7q32.2	miR-96/182/183	Thyroid, stomach, pancrease	-	KRAS(60), HTR1B(64), FOXO1(63), PRMT5(67)
miR-99a									↓	↓		↓			21q21.1	let-7c/miR-99a	Tongue, ovary, prostate	-	-
miR-122		↓	↓					↓	↓		↓	↓			18q21.3	-	Thyroid, breast	TRPV6(62), CCNG1(7), BCL2L2(66), ADAM17(69), ADAM10(61), SRF(61), IGF1R(61), CCRN4L(65)	-
miR-125a	↓		↓								↓			↓	19q13.41	let-7e/miR-99b/125a	Thyroid, breast, lung, ovary, prostate	-	ERBB2(68), ERBB3(68), TP53(183), ELAVL1(71), EDN1(74), KLF13(76)
miR-139					↓	↓						↓		↓	11q13.4	-	Tongue, pancrease	FOXO1(72)	-
miR-145		↓			↓		↓				↓			↓	5q32	miR-143/145	Blood, breast, lung, pancrease, colon, ovary, prostate	-	PARP8(73), IRS1(75), MYC(86), MUC1(85), OCT4(91), SOX2(91), KLF4(91), RTKN(89), ESR1(87), CBFB(84), PPP3CA(84), CLINT1(84), FLI1(80), YES, STAT1(79), FSCN1(77)
miR-150		↓				↓								↓	19q13.33	-	Lung, pancrease	-	MYB(83), P2RX7(92), EGR2(90)
miR-183					↑							↑		↑	7q32.2	miR-96/182/183	Blood, thyroid, lung, colon	PDCD4(82)	EZR(88), BTRC(78), ITGB1(81), KIF2A(81)
miR-199a	↓	↓	↓			↓				↓	↓	↓		↓	19p13.2 / 1q24.3	- / miR-199a-2/214/3120	Blood, throid, prostate	-	IKKB(184), HIF1A(107), SIRT1(107)
miR-199b		↓				↓	↓							↓	9q34.11	miR-199b/3154	-	-	LAMC2(73), SET(97), HES1(101)
miR-200a	↓										↓	↓			1p36.33	miR-200a/200b/429	Thyroid, ovary	-	ZEB1(102), ZEB2(102), CTNNB1(108)
miR-200b		↓				↓						↓		↓	1p36.33	miR-200a/200b/429	Thyroid, ovary	-	ZEB1(102), ZEB2(98), ERRFI1(94)
miR-210			↑							↑			↑		11p15.5	-	Thyroid, breast, lung, pancrease, colon, prostate	-	EFNA3(99), NPTX1(106), ACVR1B(105), MNT(109), CASP8AP2(104), HOXA1(103), HOXA9 (103), FGFRL1 (103), ISCU1/2(96), BDNF(100), CPEB2 (100), DDAH1(100), NCAM1(100), PTPN1(100), Xist(100), GPD1L(100), E2F3(95),
miR-214		↓			↓	↓								↓	1q24.3	miR-199a-2/214/3120	Thyroid, lung, ovary	-	PTEN(123), NRAS(122), EZH2(118), LTF(121), POU4F4(110)
miR-216a					↑					↑				↑	2p16.1	miR-216/217	Blood, nose & pharynx, pancrease	-	PTEN(119)
miR-221		↑	↑	↑	↑	↑					↑	↑	↑		Xp11.3	miR-221/222	Blood, Brain, thyroid, liver, stomach, pancrease	DDIT4(18), BMF(116), CDKN1B(113), CDKN1C (113), PTEN(115), TIMP3 (115),	KIT(112), ESR1 (124), MDM2(120), ICAM1(117)
miR-222			↑		↑			↑		↑	↑	↑	↑	↑	Xp11.3	miR-221/222	Blood, thyroid, liver ,stomach, pancrease	PPP2R2A(19), PTEN (115), TIMP3 (115),	STAT5A(111), CDKN1B (114), KIT(112), ESR1(124), MMP1(128), SOD2(128)
miR-223		↓				↓				↓		↓			Xq12	-	Stomach, pancrease, colon	STMN1(131)	NFIA(126), LMO2(127), E2F1(129)
miR-224	↑				↑			↑		↑	↑		↑	↑	Xq28	miR-224/452	Thyroid, colon, pancrease	API5(10)	Smad4 (132), KLK10(130)
miR-301a					↑	↑						↑		↑	17q22	-	Pancrease, colon, lung	-	MEOX2(125)

to be able to differentiate liver samples based on histology (hepatocellular adenoma from HCC), etiology (alcohol induced HCC from HBV associated HCC) and cancer gene mutation (β-catenin and hepatocyte nuclear factor 1α) [131]. Recently, a signature of 20 miRNAs has been shown to associate with HCC metastasis and correlates with overall survival [242]. Furthermore, high miR-221 levels in HCC were associated with tumor multifocality and reduced time to recurrence after surgery [226] and high miR-222 level negatively correlates with disease free survival [137]. These promising early studies suggest that miRNA profiling, in combination with existing clinical parameters, may provide additional information on HCC classification and prognostic risk stratification and help oncologists make better informed decisions.

The easily detectable, serum bound miRNAs are good candidates to screen for specific biomarkers to improve existing screening strategies for early HCC detection. Significant differences in blood miRNA expression can be detected and associated with well-controlled liver tumorigenesis in a mouse model [243]. In human HCC, miR-500 is reported to be elevated in the sera of HCC patients and returned to normal after surgical resection [244]. MiR-500 and others represents potential diagnostic markers for HCC detection.

As miRNAs play an important role in HCC, they represent promising therapeutic targets for novel treatment strategies for HCC patients. Exciting data have emerged in the use of antagomirs which are chemically modified oligonucleotides to specifically and effectively knock down miRNA expression. Systemic delivery of antagomirs against miR-122 has resulted remarkably specific and stable down-regulation of miR-122 in the liver up to

20 days [245]. As miR-122 promotes HCV replication, knock-down of miR-122 may provide a novel approach to manage HCV load in HCV-associated HCC. Recently Kota *et al* demonstrated the efficacy of the systemic delivery of miR-26a in an adeno-associated virus vector to protect disease progression without toxicity in a mouse liver cancer model [246-248], opening new doors to miRNA replacement therapy.

1.3 Aims and significance of this study

At the time when this project started, microRNAs were only starting to gain recognition as functional molecules with meaningful biological functions. MicroRNA deregulation was first reported in acute myeloid leukemia [43, 44] but very little was known about its status in human HCC. Therefore, in this thesis, we aim to:

1. Profile miRNA expression of tumor versus adjacent non-tumor samples from local HCC patients to identify significantly differentially expressed miRNAs in HCC.
2. To functionally characterize these significantly differentially expressed miRNAs to understand their roles in HCC.
3. To identify the cellular targets through which these deregulated miRNAs impact cellular processes.

We hope our work will contribute to improve the understanding of HCC from a fresh perspective of miRNA deregulation and offer novel approaches to treat this complex disease.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines

The immortalized untransformed human liver cells, THLE3, was purchased from American Type Culture Collection (ATCC) and cultured in ATCC complete growth medium consisting of BEGM® Bronchial Epithelial Cell Growth Medium (CC-3170, Lonza) without gentamycin/amphotericin (GA) and epinephrine, supplemented with additional 10 ng/ml epithelial growth factor (EGF), 200 ng/ml O-phosphoethanolamine and 10% fetal bovine serum (FCS, Bio-industries). THLE3 cells were grown on surfaces precoated with rat tail collagen type I (BD).

The human hepatocellular carcinoma cells, HepG2 and SNU-182, and the human embryonic kidney cells, HEK293, were purchased from ATCC and cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 25 mM HEPES (Invitrogen) and 10 % FCS. The human colorectal carcinoma cells, HCT116, was purchased from ATCC and cultured in Modified McCoy's 5A Medium (Sigma) supplemented with 2.2 g/L sodium bicarbonate and 10 % FCS. All cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2.

2.2 Patient samples

Paired tumorous and adjacent nontumorous liver tissues from 100 hepatocellular carcinoma patients were obtained from the National Cancer Centre of Singapore Tissue Repository with prior approval from the National

Cancer Centre (NCC) Institutional Review Board (IRB) (NCC IRB No: NC08-12). As it is ethically not possible to obtain normal livers from healthy individuals, the 'normal' non-tumorous portion of the livers from 40 colorectal cancer patients with metastasis to the liver was obtained when these patients undergo surgery to remove the colorectal metastatic tumor in the liver. These tissues were also obtained from the National Cancer Centre of Singapore Tissue Repository with prior approval from the National Cancer Centre (NCC) Institutional Review Board (IRB) (NCC IRB No: NC05-022). These non-tumorous livers from colorectal cancer patients represent 'normal' liver in our study.

2.3 Total RNA extraction

Total RNA including low molecular weight (LMW) RNAs were extracted with mirVana^{TM} microRNA Isolation Kit (Ambion) following manufacturer's instructions. Briefly, 1x10^6 cells or 0.3 g of homogenized patient tissues were lysed in 500 µl of lysis buffer and 50 µl of miRNA homogenate additive and incubated on ice for 10 minutes. The lysate were then passed through a collection column. The columns were washed once with 600 µl of Wash Buffer 1, twice with 500 µl Wash Buffer 2/3 and once with 700 µl of 80 % ethanol in nuclease free water, by centrifuging briefly at 10,000 rpm. The columns were then dried by centrifuging at 13,000 rpm for an additional minute and the total RNA including LMW RNAs were then eluted with 50 µl of nuclease free water preheated at 99 °C.

2.4 microRNA expression profiling in HCC patients

Total RNA extracted from tumor and paired adjacent non-tumor tissues from 117 HCC patients were first checked for quality using the Agilent 2100 Bioanalyzer. RNA Integrity Number (RIN) was calculated for each sample. A RIN number of 10 indicates high RNA quality, and a RIN number of 1 indicates low RNA quality. According to published data, RNA with a RIN number > 6 is of sufficient quality and 100 out of 117 pairs of HCC samples were processed for miRNA microarray experiments[249]. The HCC tumor samples were labeled with Hy5 (red) and the paired adjacent non-tumor samples were labeled with Hy3 (green) according to the undisclosed miRXplore™ user manual. Subsequently, the fluorescently labeled samples were hybridized overnight to miRXplore™ Microarrays using the a-Hyb™ Hybridization Station. Fluorescence signals of the hybridized miRXplore™ Microarrays were detected using an Agilent laser scanner. Mean signal and mean local background intensities were obtained for each spot of the microarray images using the ImaGeneâ software (Biodiscovery). Low-quality spots were flagged and excluded from data analysis. Unflagged spots were analysed with the PIQOR™ Analyzer software. The PIQOR Analyzer allowed automated data processing of the raw data text files derived from the ImaGene software. This included background subtraction to obtain the net signal intensity, data normalization, and calculation of the Hy5/Hy3 ratios for the species of interest. As an additional quality filtering step, only spots/genes were taken into account for the calculation of the Hy5/Hy3 ratio that have a signal that was equal or higher than the 50 % percentile of the background signal intensities. The above steps were performed by Miltenyi Biotec

microarray servicing facility in Germany and normalized ratios of tumor vs paired adjacent non-tumor samples were returned to us for further analysis.

Significance Analysis of Microarrays (SAM) [250] was then utilized to identify miRNAs that displayed significantly differential expression between the tumor and adjacent non-tumorous liver tissues of HCC patients, at a false discovery rate (FDR) of less than 0.05 and an absolute SAM score of more than 2.5.

2.5 Validation of let-7 down-regulation and miR-224 up-regulation in HCC patients

2.5.1 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The expression of specific miRNAs identified to be significantly differentially expressed in HCC was validated with Taqman-based RT-qPCR using Taqman MicroRNA Individual Assays (hsa-let-7a, hsa-let-7b, hsa-let-7c, hsa-miR-224, RNU48, Applied Biosystems). Reverse transcription reaction was carried out on 75 ng of template total RNA with the High Capacity cDNA Archive Kit (Applied Biosystems) and miRNA specific reverse transcription primers. The reaction mixture was incubated at 16 °C for 20 minutes for primer annealing and this was followed by reverse transcription at 42 °C for one hour, deactivation at 85 °C for 5 minutes and pause at 25 °C. Real-time PCR was performed in a 10 µl reaction mix comprising 2 µl of 2X diluted reverse transcription product, 5 µl of Taqman 2X Universal PCR Master Mix without UNG Amperase, 2 µl of miRNA specific probes and primers and 1 µl

of nuclease free water, on an Applied Biosystems 7500 Real Time PCR system, with an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Fluorescence signal was measured at each extension step.

2.5.2 Northern blot analysis

Northern blot analysis was performed to further validate miR-224 over-expression in the tumors of HCC patients. Briefly, 1 μg of total RNA (HCT116 cells transfected with Pre-miR^{TM} miR-224 precursor as positive controls) or 2 μg of low molecular weight RNA from both the tumor and adjacent non-tumorous tissues of selected HCC patients that were previously profiled was separated on a 15 % denaturing polyacrylamide gel and electro-blotted onto a nylon membrane (Schleicher & Schuell GmbH, Dassel Germany) at 300 mA for 30 minutes. The miRNA-224 probe (5'-TAAACGGAACCACTAGTGACT TG-3' [62]) and U6 probe (5'-ATGTGCTGCCGAAGCGAGCAC -3') were end-labeled with Redivue γ-^{32}P ATP (Amersham Biosciences, UK) using T4 Polynucleotide Kinase (NEB, Ipswich, MA) and purified using the Nucleotide Removal Kit according to the manufacturer's instructions (Qiagen GmbH, Germany). Hybridization was performed using Express Hybridization solution (Clontech, Mountain View, CA) at 42 °C for 16 hours, and the blots were exposed to Hyperfilm MP (Amersham Biosciences, UK) and developed in a Biomax machine (Kodak, USA).

2.6 Western blot quantitation of hepatitis B virus protein X (HBx) status in HCC patients

HBx protein level in tumor and non-tumor samples from HCC patients was measured through Western blot analysis with a specific rabbit anti-HBx polyclonal antibody generated against full-length HBx by our lab. Briefly, tissue samples from HCC patients were first homogenized and lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl pH 7.0, 1 % Deoxycholate, 1 % NP40, 0.1 % SDS) buffer supplemented with protease inhibitor cocktail (Roche, Germany), sonicated at 30 seconds on and 30 seconds off for a total time of 10 minutes in a Bioruptor (Diagenode, Belgium) with high settings and centrifuged at 13,000 rpm for 5 minutes to collect total cell lysate. 20 μg of total protein lysates were resolved on a 12 % SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 2 % ECL^{TM} advanced blocking agent (GE health) in 1X phosphate buffered saline with 0.1 % Tween-20 (PBST) for one hour and probed with rabbit-anti-HBx at a dilute of 1:10,000 and goat-anti-β-actin 1:40,000 (Santa Cruz, USA) and horseradish peroxidase (HRP) conjugated goat-anti-rabbit or rabbit-anti-goat secondary antibody, respectively, at a dilute of 1:100,000 (Pierce). The blots were then washed and visualized with Enhanced Chemiluminescence Reagent Kit (ECL, Amersham Bioscience) in a Biomax machine (Kodak, USA). The intensity of the HBx protein Western blot signal was quantified with AlphaImager 2000 software and normalized against that of beta-actin of the same sample. Relative fold change of HBx was calculated as the ratio between the normalized HBx expression in tumor and non-tumor samples in Log2 scale.

2.7 Recombinant adenovirus propagation, titration, optimization and transduction

Recombinant adenovirus carrying the full length HBx gene (AdHBx) and the control adenovirus (AdControl) was previously constructed in our lab and reported to achieve > 90 % transduction efficiency with minimal cytotoxicity [251] (Figure 2.7A).

2.7.1 Virus propagation

AdHBx and AdControl recombinant adenovirus were propagated in the packaging HEK293 cells. Briefly, HEK293 cells were seeded into 175 cm^2 tissue culture flasks and grown to ~70 % confluency. Stock AdHBx or AdControl viruses were used to infect these HEK293 cells at a multiplicity of infection (MOI) ratio of 1:1. 72 hours post transduction, infected cells were harvested and suspended in 1 ml PBS per 175 cm^2 flask. Total cell content containing the propagated viruses was extracted by three repeated cycle of freezing in liquid nitrogen and thawing at 37 °C and centrifuged at 10,000 g to remover cell debris. The supernatant was the freshly prepared virus stock and stored at -80 °C in 100 µl aliquots.

2.7.2 Virus titration

HEK293 cells were seeded into 24 well plates and grown to 60 % confluency. Freshly prepared AdHBx or AdControl virus stock were serially diluted to 10^{-5} to 10^{-9} per ml and used to transduced the HEK293 cells in 4 wells for each dilution and 4 wells were left untransduced. The titer of the

virus (expression-forming units/ml) was evaluated by counting the number of fluorescent cells 48 hours after transduction with serially diluted viruses.

2.7.3 Virus transduction optimization

HepG2 cells were seeded into 6 well plates and grown to 60 % confluency. Appropriately titrated AdHBx or AdControl viruses were used to transduce HepG2 cells at MOI of 0, 1, 2, 5, 10 and 25 virues to each cell. Transduction efficiency was assessed as the percentage of green fluorescent cells with respect to the total number of cells, as seen from a fluorescence microscope, 48 hours post transduction (Figure 2.7B). Cytotoxicity was assessed by comparing the cell death and cell growth of infected cells at various MOI to the uninfected cells. Western blot analysis with specific rabbit-anti-HBx (1:10,000), goat-anti-beta-actin (1:40,000) and mouse-anti-enhanced green fluorescent protein (EGFP) (1:40,000) were used to assessed the expression of HBx and EGFP in the infected cells. Best MOI of 10:1 for AdHBx virus was determined as one at which there is significant HBx expression with > 90 % transduction efficiency and minimal cytotoxicity. Best MOI of 5:1 for AdControl virus was determined as one at which similar level of EGFP is observed in AdControl cells with similar transduction efficiency, compared to the AdHBx cells with the optimized MOI.

2.7.4 Virus transduction for miRNA microarray

HepG2 cells were transduced with AdHBx or AdControl recombinant adenovirus at a MOI of 10:1 and 5:1, respectively. Cells were harvested 72 hours post transduction. Total RNA was extracted as previously described (Section 2.3) for miRNA microarray and down-stream studies.

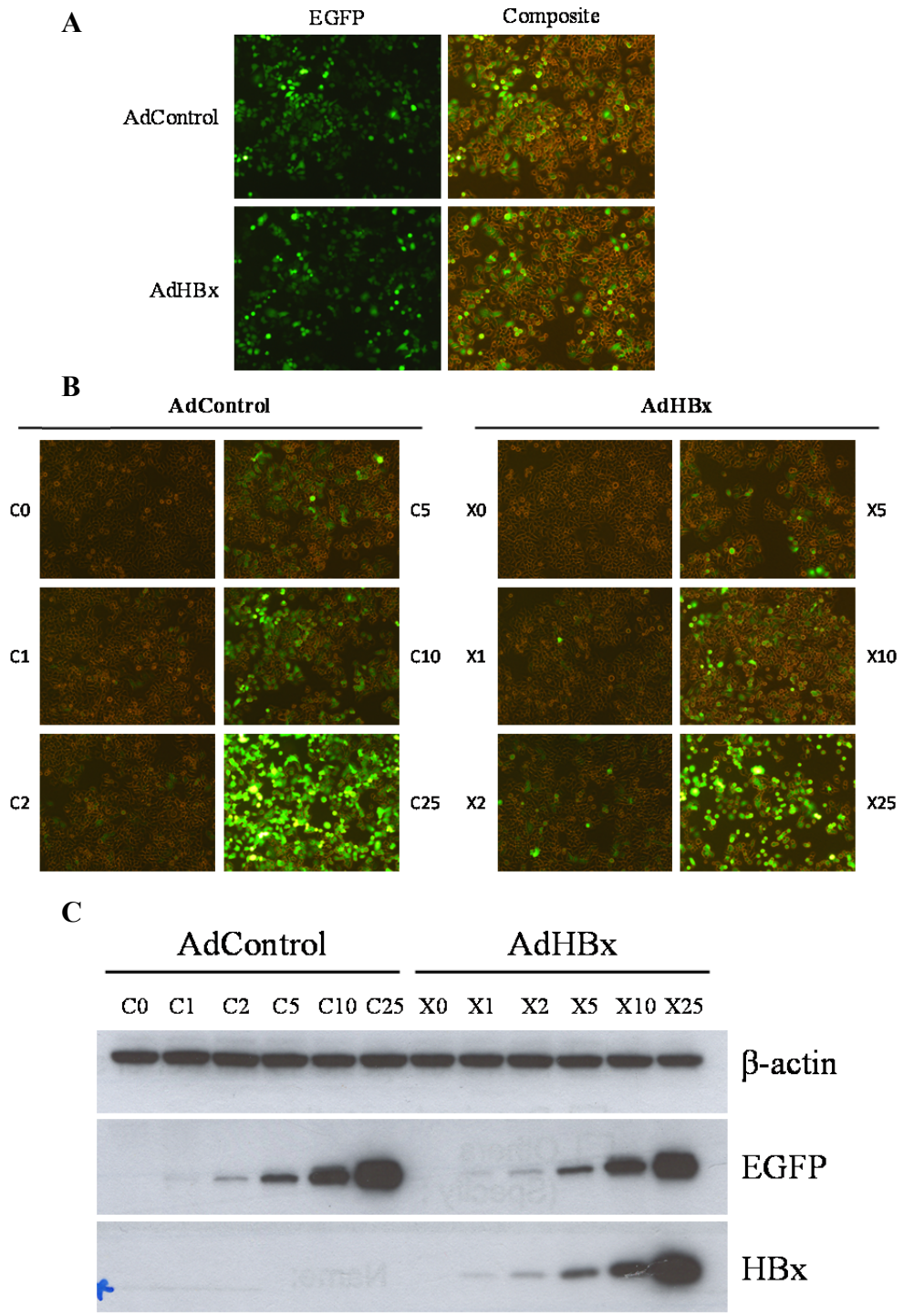


Figure 2.7 Optimization of recombinant adenovirus transduction. (A) Recombinant adenovirus can achieve high transduction efficiency with minimal cytotoxicity. **(B)** Composite image of HepG2 cells transduced with recombinant adenovirus (AdControl or AdHBx) at various MOI as indicated. "C" denotes AdControl and "X" denotes AdHBx. The number denotes the MOI used. **(C)** Western blot analysis of EGFP, HBx and β-actin in HepG2 cells transduced with AdControl or AdHBx at various MOI.

2.8 microRNA expression profiling in HBx expressing HepG2 cells

A total of 286 human microRNAs were examined between AdControl and AdHBx transduced HepG2 cells, using Ambion mirVana™ Bioarray V2 version 7.1 following manufacturer's instructions (Ambion, USA). Briefly, low molecular RNA were enriched from 20 μg of total RNA samples in a FlashPAGE™ fractionator (Ambion, USA) and the <40 nucleotide-long RNA were recovered with sodium acetate/ethanol and precipitated at -20 °C overnight. The enriched low molecular RNA from HBx-expressing samples were labeled with Cyanine-5 (Cy-5) while that from the control samples were labeled with Cyanine-3 (Cy-3) with ULS™ Small RNA Labeling Kit according to manufacturer's instructions (Kreatech, Netherlands). The Cy-5 and Cy-3 labeled samples were then mixed in equal amount and hybridized onto the mirVana™ bioarrays at 42 °C overnight. The hybridization signals were detected in dual colours with a GenePix 4000B scanner and quantitated with GenePix Pro 6.0 (Molecular Devices, Canada). The miRNA expression in HBx- versus control cells were profiled in three independent experiments using three separate bioarrays. Raw data from the two-colour miRNA microarrays were normalized with locally weighted scatterplot smoothing (LOWESS) algorithms with GeneSpring GX Software version 7.3 (Agilent Technologies). Significantly expressed miRNAs were identified as those with foreground signal intensity greater than the background intensity plus 3 standard deviations. Significantly differentially expressed miRNAs between HBx-expressing cells and control cells were determined using a Volcano Plot with threshold set at absolute mean fold change of greater than 1.5 and p-value of less than 0.01.

2.9 Transfection of small oligos into cell lines

2.9.1 Chemical transfection

HCT116 or THLE3 cells were seeded into 6 well plates and grown to 50% confluency. siPORT^{TM} Amine Transfection Reagent (Ambion) was used to transfect small oligos such as Control Oligos, miRNA precursors and miRNA inhibitors in a final concentration of 30 nM according to manufacturer's recommendations. Table 2.9 listed the small oligos used in this study. Briefly, 9 µl of siPORT Amine were mixed with 1ul of 50 uM stock oligos in 200 µl of OptiMEM I (Invitrogen) medium and incubated at room temperature for 30 mins. These 210 µl reaction mixture was then added into individual wells containing 1.5 ml of normal growth medium each and incubated overnight.

2.9.2 Electroporation

$2x10^6$ HepG2 cells were electroporated with 50 nM of Control oligos, let-7a precursor, let-7a inhibitor or siRNA against STAT3 in 200 µl of OptiMEM I (Invitrogen, USA) at 175 V for 100 ms in a BTX Electro Square Porator 830 (BTX, USA). Cells were harvested 24 hours post electroporation for downstream analysis.

Table 2.9 List of short oligos used in transfection or electroporation.

Oligo Name	Commercial name	Catalogue number	Company
Control Oligos 1	Scramble-miR; Control, miRCURY™ knockdown probe	199002-00	Exiqon, Denmark
let-7a precursor	Ambion Pre-miR™ hsa-let-7a Precursor Molecules	PM10050	ABI, USA
let-7a inhibitor	miRCURY LNA™ hsa-let-7a knockdown	118000-00	Exiqon, Denmark
Control Oligos 2	Pre-miR™ miRNA Precursor Molecules—Negative Control #1	AM17110	ABI, USA
miR-224 precursor	Ambion Pre-miR™ miR-224 Precursor Molecules	PM12571	ABI, USA
miR-224 inhibitor	Ambion Anti-miR™ miR-224 inhibitor	AM12571	ABI, USA
si-STAT3	Validated MISSION® siRNA	SASI_Hs01_00121206	Sigma, USA
si-Smad4	MISSION® siRNA	SASI_Hs01_00207793	Sigma, USA

2.10 Generation of 3'UTR reporter constructs

The 1080 base pairs of wild type STAT3 3'UTR was amplified from human genomic DNA and cloned downstream a β-galactosidase reporter gene driven by multidrug resistance associated gene (MRP1) promoter at the SacII restriction site (Figure 3.2.5.(1)A). The human MRP1 promoter was chosen over the constitutive human cytomegalovirus (CMV) promoter, because the MRP1 promoter is ~30 times weaker than the CMV promoter which will facilitate the measurement of subtler changes in reporter gene activity. The reporter construct also carried a CMV promoter driven enhanced green fluorescence protein (EGFP) to normalize for differences in transfection efficiencies. The recombinant reporter vector carrying the wildtype STAT3 3'UTR was termed pSTAT3-3UTR-WT. A mutant pSTAT3-3UTR-MUT, where the putative let-7 binding site was mutated, was also generated by PCR mutagenesis. The cloning strategies and primers used were listed in Figure 2.10.1A&B. The mutant site was verified *in silico* not to bind to known human miRNAs using miRBase (Release 12.0). Both constructs were verified by sequencing (Figure 2.10.1C).

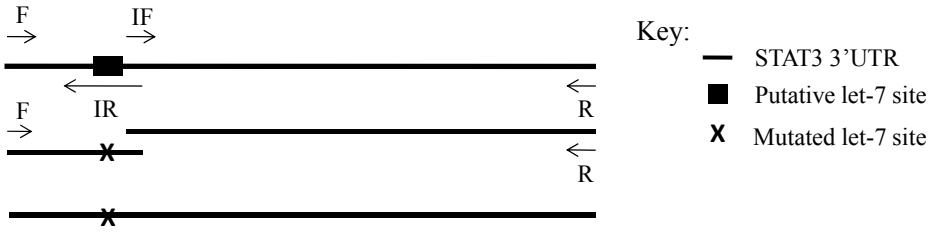
As outlined in Figure 2.10.2, using a similar strategy, the 2015 bp wildtype API5 3'UTR was amplified from human genomic DNA and cloned into the same backbone β-galactosidase reporter construct (pAPI5-3UTR-WT) as described above (Figure 3.3.5.(1)A). A mutant pAPI5-3UTR-MUT was also generated by PCR mutagenesis where three point mutations were generated on each of the three miR-224 target recognition sites. The 1310 bp wildtype SMAD4 3'UTR was amplified from non-tumorous human liver

tissue and cloned into the reporter construct (pSMAD4-3UTR-WT) (Figure 3.3.8.(1)A). A mutant pSMAD4-3UTR-MUT, where the two putative miR-224 binding sites were abolished through point mutations, was also generated by PCR mutagenesis. All mutant recognition sites were verified *in silico* not to bind to any of the known human miRNAs using miRBase. All reporter constructs generated were confirmed by sequencing.

2.11 Characterization of miRNA interaction with putative binding sites on target gene 3'UTR through 3'UTR β-galactosidase assay

HCT116 cells were transfected in 6-well plates by using siPORT™ Amine Transfection Agent (Ambion, Austin, TX) according to the manufacturer's instructions with either 1.0 μg of the β-gal reporter construct containing the wild type target gene 3'UTR sequences (pSTAT3-3UTR-WT, pAPI5-3UTR-WT or pSMAD4-3UTR-WT), or the corresponding β-gal reporter construct containing the mutant target gene 3'UTR sequences (pSTAT3-3UTR-MUT, pAPI5-3UTR-MUT or pSMAD4-3UTR-MUT); and co-transfected with either 30 nM of Control Oligos or miRNA precursors (Ambion Pre-miR^{TM} hsa-let-7a or hsa-miR-224 precursor molecules). β-gal reporter gene activity was assayed kinetically using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate and measured at 1 minute intervals over 60 minutes at 570 nm in a SpectraMAX PLUS microplate reader (Molecular Devices, Sunnyvale, California, USA) with crude lysate from the transfected cells harvested 24 hours post transfection.

A



B

Primers	Sequence
F	GTGACCGCGGGGAGCTGAGAACGGAAGCTG
IR	TCAAAGATAGCATGTTGGTTCTCGGTAGAGCAATCTGGAACCACAAAGTTAG
IF	GCTCTACCGAGAACCAACATGCTATCTTTGAGCAATCTGGG
R	CTAACCGCGGAATGCTTAGATTCTCCTTAAACC

C

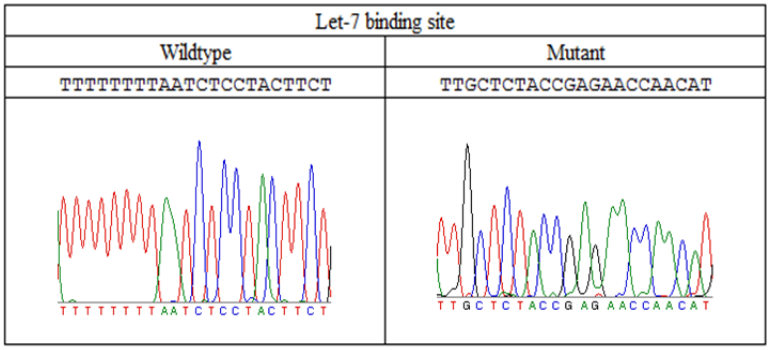
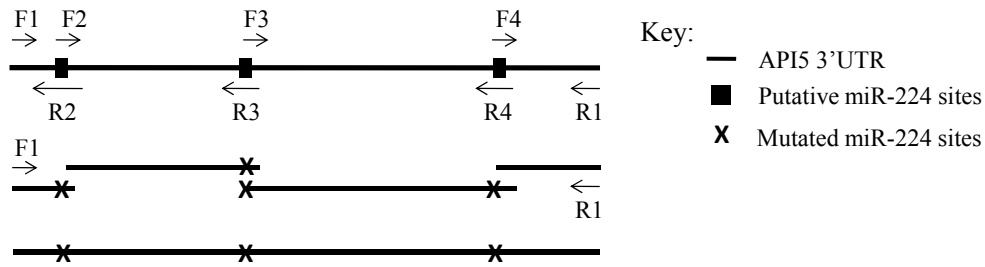


Figure 2.10.1 Generation of STAT3 3'UTR reporter constructs. (**A**) Strategy for the generation of mutations at the let-7 binding site of STAT3 3'UTR. **(B)** Primers used for the generation of mutations at the let-7 binding site of STAT3 3'UTR**. (C)** Product of the mutagenesis that was used to clone into the β-gal reporter construct and sequence confirmed.

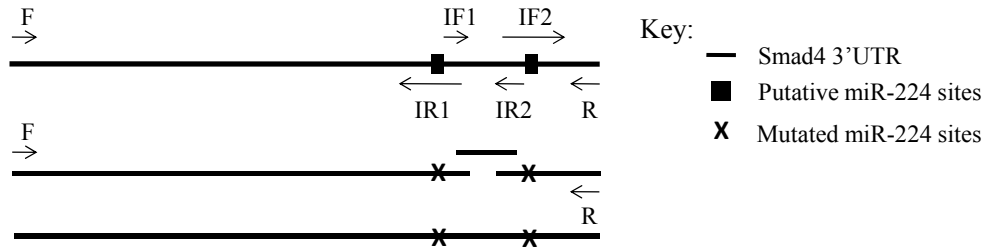
A



B

Primers	Sequences
API5_F1	5'-TATACCGCGGGGTTGGGGCACACGAGGAAA-3'
API5_F2	5'-ATGGTTTTTGCAGTATCATTATTCA-3'
API5_F3	5'-AGAAAAAGTTAATGGATAACATGGTA-3'
API5_F4	5'-AGCAGAAGGGCCGTTACTTTCTCA-3'
API5_R1	5'-TATACCGCGGGCATAAGGGGACATTTACATTTAA-3'
API5_R2	5'-GAATAATGATACTGCAAAAACCATTAAAGATT-3'
API5_R3	5'-TACCATGTTATCCATTAACTTTTTCAGGGTA-3'
API5_R4	5'-AGAAAGTAACGGCCCTTCTGCTCAGT-3'

C



D

Primers	Sequences
Smad4_F	5'-GACTGACCGCGGTCTTTTACCGTTGGGGCCCTTAACC-3'
Smad4_R	5'-ATGGTTTTTGCAGTATCATTATTCA-3'
Smad4_IF1	5'-TTTTTGGTATAATGTTTAAATCATG-3'
Smad4_IR1	5'-CATGATTTAAACATTATACCAAAAATGTAGGTTTCAAAGTAAGTACAAAAATATCCAAACTACCTATTATG-3'
Smad4_IF2	5'-GCCCTTTGCCATCAATGATCATATCTCTTGGCTACCCTACTGTATAGAGAATTTAAGTAG-3'
Smad4_IR2	5'-GATATGATCATTGATGGCAAAGGGCT-3'

Figure 2.10.2 Generation of API5 and SMAD4 3'UTR reporter constructs. **(A)** Strategy for the generation of mutations at the miR-224 binding site of API5 3'UTR. **(B)** Primers used for the generation of mutations at the miR-224 binding site of API5 3'UTR**. (C)** Strategy for the generation of mutations at the miR-224 binding site of SMAD4 3'UTR. **(D)** Primers used for the generation of mutations at the miR-224 binding site of SMAD4 3'UTR**.**

To normalize for differences in transfection efficiencies, Western blot analyses were performed using mouse anti-EGFP (Roche Diagnostics) at 1:40,000 dilution and HRP-conjugated goat anti-mouse (Pierce Biotechnology) secondary antibody at 1:100,000. β-gal activity was then normalized against EGFP expression levels. The data was also normalized against differences in basal β-gal activity of each construct in the absence of any miRNA oligos.

2.12 Functional assays

2.12.1 Apoptosis assay

HepG2 cells were transduced with recombinant HBx or control adenovirus as mentioned above. 48 hours post transduction, the Control cells or HBx cells were treated with either 20μM of control inhibitor (z-FA-FMK, 550377,BD Pharmingen) or 20μM of general caspase inhibitor (z-VAD-FMK, 550377, BD Pharmingen) for an additional 24 hours. The apoptotic profile of these treated cells were then assayed with PE-Annexin V Apoptosis Kit I (BD Bioscience) according to manufacturer's protocol and analyzed with FACS-Calibur flow cytometer (BD Biosciences). Apoptotic cells were represented by high PE-Annexin V and low 7-AAD fluorescence signals.

HCT116 cells were transfected using siPORT^{TM} Amine Transfection Agent with either 60 nM Control or 30 nM miR-224 precursor and 30 nM Control or 30 nM miR-224 precursor and 30 nM Anti-miR^{TM} miR-224 inhibitor (miR-224 inhibitor (AM17000, Ambion). Apoptosis assay was performed 48 hours post transfection as described above.

2.12.2 Cell proliferation assay

Cell proliferation of HepG2 cells transfected let-7a precursors or transduced with recombinant adenoviral vectors was measured with Cell Proliferation Reagent (WST-1) (Roche, Germany) according to supplier's protocol. Briefly, 10,000 treated cells were seeded in a 96-well flat bottom tissue culture plate and incubated with WST-1 for 2 hours. Cell proliferation was measured as the absorbance at OD_{440} in a VersaMax^{TM} Microplate Reader (Molecular Devices, USA).

Cell proliferation of HCT116 cells transfected with miR-224 precursors was assayed using the BrdU cell Proliferation Assay kit (Calbiochem, San Diego, USA) following the manufacturer's protocol. BrdU incorporation measured as absorbance at OD_{450} in a SpectroMAX PLUS microplate reader (Molecular Devices, Sunnyvale, California, USA) 48 hours after BrdU incubation.

2.13 Reverse transcription quantitative real-time PCR analysis

The differential expression of mature miRNAs was validated using Taqman MicroRNA Individual Assays (Applied Biosystems). Each sample was analyzed in triplicates. Reverse transcription reaction was carried out on 75 ng of template total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems) and miRNA specific reverse transcription primers . Real-time PCR was performed in a 10 µl reaction mix comprising 2 µl of 2X diluted reverse transcription product, 5 µl of Taqman 2X Universal PCR

Master Mix without UNG Amperase, 2 µl of miRNA specific probes and primers and 1 µl of nuclease free water, on an Applied Biosystems 7500 Real Time PCR system, with an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Fluorescence signal was measured at each extension step. The Taqman microRNA individual assays used in this study are listed in Table 2.13 (Top)

Target gene transcript expression was analyzed with QuantiTect™ SYBR Green PCR Kit (Qiagen). cDNA was synthesized from 2 µg of total RNA with the High Capacity cDNA Archive Kit (Applied Biosystems) and a poly-T containing primer (RT reverse, 5'- GGCCACGCGTCGACTAGTAC TTTTTTTTTTTTTTTT-3'). Real-time PCR was performed on an Applied Biosystems 7500 Real-time PCR Machine. Amplification reaction mix included cDNA template (25 ng), target gene primers (0.25 µM), and 2X PCR Mater Mix (5 µl, Qiagen) in a total volume of 10 µl. Amplification conditions included an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 33 seconds. SYBR Green fluorescence was measured at each extension step. The specific primer sequences for each target gene examined are listed in Table 2.13(Bottom).

The level of transcript expression was measured by threshold cycle (CT), which was determined as the fractional cycle number at which the fluorescence intensity exceeded a fixed threshold, and the ΔΔCT method was employed for relative quantitation of gene expression [252]. The normalized CT (ΔCT) was calculated by subtracting the CT of an endogenous control from the CT of gene of interest. The ΔΔCT was calculated by subtracting the

Table 2.13 List of Taqman microRNA individual assays (Top) and primer sequences (Bottom) used in RT-qPCR

miRNA ID	Part Number
hsa-let-7a	4373169
hsa-let-7b	4373168
hsa-let-7c	4373167
hsa-let-7d	4373166
hsa-let-7e	4373165
hsa-let-7f	4373164
hsa-let-7g	4373163
hsa-let-7i	4373162
hsa-miR-98	4373009
hsa-miR-224	4373187
RNU48	4373383

Gene Name		Sequences
API5	F	5'-TAGTGGGTTTGGAGAAGTTC-3'
	R	5'-TCACTTGATAGGCATCTTTATG-3'
STAT3	F	5'-CAGGAGGGCAGTTTGAGTCC-3'
	R	5'-CAAAGATAGCAGAAGTAGGAGA-3'
Smad4	F	5'-AGGATCAGTAGGTGGAATAG-3'
	R	5'-TCTAAAGGTTGTGGGTCTGC-3'
U6	F	5'-CTCGCTTCGGCAGCACA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'
CDKN1A	F	5'-GATGAGTTGGGAGGAGGCAG-3'
	R	5'-TTGGAGTGGTAGAAATCTGTCA-3'
Beta-actin	F	5'-ATGTTTGAGACCTTCAACACC-3'
	R	5'-AGGTAGTCAGTCAGGTCCCGGCC-3'

ΔCT of the control sample from that of the treated samples. The fold change was calculated with the equation $2^{-\Delta\Delta CT}$.

2.14 Western blot analysis

Cells were lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl pH 7.0, 1% Deoxycholate, 1% NP40, 0.1% SDS) lysis buffer supplemented with protease inhibitor cocktail (Roche, Germany), sonicated for 10 minutes in a Bioruptor (Diagenode, Belgium) and centrifuged at 13,000 rpm for 5 minutes to collect total cell lysate. Protein lysates were resolved on a 12 % SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane. The blots were blocked with 2 % ECL^{TM} advanced blocking agent (GE health) in 1X PBST for one hour and probed with respective primary antibody and horseradish peroxidase conjugated secondary antibody. The primary and secondary antibodies were diluted as indicated in Table 2.14. The blots were then washed and visualized with Enhanced Chemiluminescence Reagent Kit (ECL, Amersham Bioscience) in a Biomax machine (Kodak, USA).

Table 2.14 List of antibodies used in Western blot analysis

Primary antibody	Source	Type	Company	Catalogue number	Dilution Factor
HBx	Rabbit	Polyclonal	Custom made	-	1:10000
STAT3	Mouse	monoclonal	Santa Cruz	SC-8019	1:1000
MYC	Mouse	monoclonal	Santa Cruz	SC-142	1:2000
EGFP	Mouse	monoclonal	Roche	11814460001	1:40000
Smad4	Mouse	monoclonal	Santa Cruz	SC-56479	1:2000
Beta-actin	Goat	Polyclonal	Santa Cruz	SC-1646	1:40000

Secondary antibody	Conjugation	Type	Company	Catalogue number	Dilution Factor
Goat-anti-Mouse	HRP	Polyclonal	Pierce	31430	1:100000
Goat-anti-Rabbit	HRP	Polyclonal	Pierce	31460	1:100000
Rabbit-anti-Goat	HRP	Polyclonal	Pierce	31402	1:100000

2.15 Statistical analyses of experimental data

Unpaired two-tailed t test was performed to analyze the significance of differences between sample means obtained from at least three experiments. Pearson correlation was used to analyze the relationship between the HBx protein expression and the let-7 miRNA expression, between miR-224 expression and API5 transcript expression, between miR-224 expression and SMAD4 transcript expression, in HCC patient samples.

CHAPTER 3: RESULTS

3.1 miRNA expression profiling identifies dysregulation of miRNAs that are associated with HCC.

To identify deregulated miRNAs in HCC, we examined the expression profile of 859 human mature miRNAs/miRNA* in 100 HCC tumor versus the paired adjacent non-tumor tissues using miRXplore microRNA microarrays (miRBase release 12). Of these 859 miRNAs, 267 miRNAs were not expressed in samples from HCC patients and 411 miRNAs were expressed in less than 10 % of HCC samples. Only a total of 181 miRNAs were found to be significantly expressed in more than 10 patient samples. Only these miRNAs were included in subsequent analysis. Significance Analysis of Microarrays (SAM) was used to identify statistically significantly deregulated miRNAs in HCC. A total of 66 miRNAs were identified to be significantly differentially expressed between tumor versus adjacent non-tumor samples in HCC patients with a threshold of false discovery rate (FDR) < 0.000 and absolute d score ($|d| > 3$).

Figure 3.1 is a Heat Map showing these 66 significantly differentially expressed miRNAs, 50 of which were up-regulated (red) and 16 were down-regulated (green). Using unsupervised hierarchical clustering analysis with Pearson correlation and average linkage, distinct clusters of miRNAs were observed to be associated with either functional families: let-7 family (let-7a, let-7b and let-7c, in yellow box), miR-15 family (miR-15a and miR-15b, in orange box), miR-221 family (miR-221 and miR-222, in light blue box), miR-320 family (miR-320b, miR-320c and miR-320d, in pink box) and miR-199A

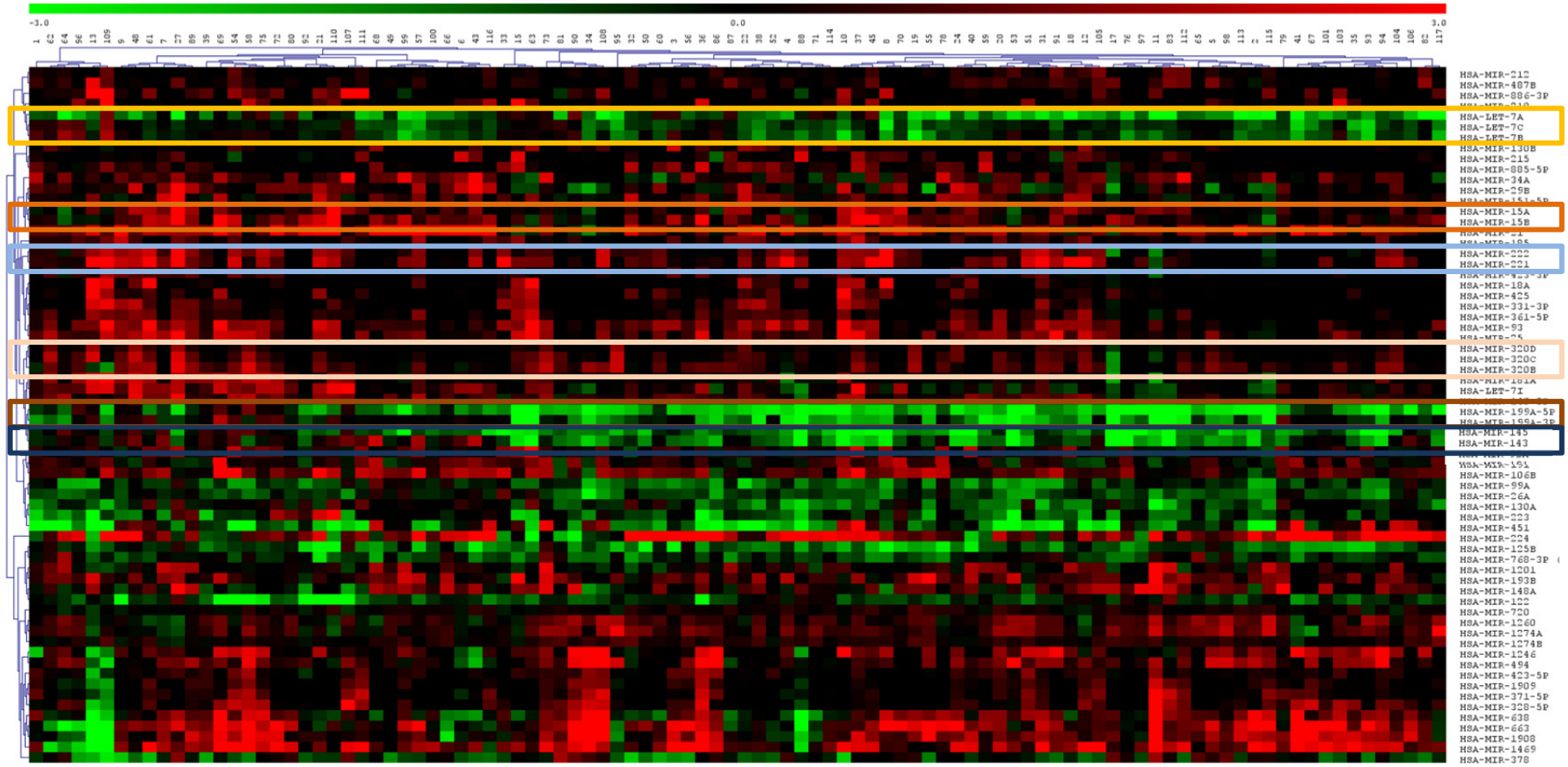


Figure 3.1 Significantly differentially expressed miRNAs between tumor and paired adjacent non-tumor samples from 100 HCC patients. The different HCC patients are represented on the Y-axis and the results are presented as mean fold change in miRNA expression of tumor versus adjacent non-tumorous tissue in each patient (X-axis). Red represents miRNAs that are over-expressed in the tumors while green represents miRNAs whose expression is down-regulated in tumors. Orange boxes indicate the miRNA cluster shown from unsupervised hierarchical clustering.

(Unpublished data)

family (miR-199A-5P and miR-199A-3P, in brown box), or associated with genomic loci: miR-221/222 genomic cluster on ChrXp11.3 (in light blue box) and miR-143/145 genomic cluster on Chr5q32 (in dark blue box). These data suggests that these miRNA clusters may be coordinately regulated base on either the family-defining seed sequences or the genomic loci.

As shown from Table 3.1 which annotated these significantly deregulated miRNAs based on genomic loci, these 66 differentially expressed miRNAs were distributed across all human chromosomes except Chromosomes 4, 6, 10 and 20, with a slight enrichment in Chromosome 1 (6 miRNAs), Chromosome 3 & X (6 miRNAs each) and Chromosome 7, 11, 17 & 19 (5 miRNAs each). 32 miRNAs (~50 %, top half of Table 3.1) were found in miRNA clusters on the human genome and many of these miRNA clusters showed evidence of co-regulation at these genomic loci. For example, let-7a-3/7b cluster on Chr22q13.31 may account for the coordinated down-regulation of let-7a and let-7b in HCC tumors. Similarly, miR-25/93/10b cluster on Chr7q22.1 may be responsible for the coordinated up-regulation of miR-25, miR-93 and miR-106b. A total of 7 such miRNA clusters (shaded in light green and pink) can be identified to potentially account for coordinated regulation of 16 miRNAs out of a total of 32 miRNAs found in genomic clusters, suggesting the regulation of genomic loci as a common mechanism of regulationg miRNA expression. Besides the two miRNA clusters mentioned, the other miRNA clusters include miR-99a/let-7c on Chr21q21.1 (miR-99a and let-7c down-regulation), miR-17-92 cluster on Chr13q31.3 (miR-18a and miR-92a up-regulation), miR-143/145 cluster on Chr5q32 (miR-143 and miR-145 down-regulation), miR-191/425 cluster on Chr3p21.31 (miR-191 and

Table 3.1 List of differentially expressed miRNAs in HCC with genomic and functional annotations.

miRNAs	Murakami *et al* 2006	Gramantieri *et al* 2007	Meng *et al* 2007	Huang *et al* 2008	Wang *et al* 2008	Jiang *et al* 2008	Varnholt *et al* 2008	Ladeiro *et al* 2008	Connolly *et al* 2008	Su *et al* 2009	Huang *et al* 2009	Ura *et al* 2009	Pineau *et al* 2010	Wong *et al* 2010	**Thesis**	Genomic loci	Genomic Cluster	Functional family
let-7a		↓		↑					↓		↓				↓	9q22.23 11q24.1 22q13.31	let-7a-1/7f-1/7d miR-100/let-7a-2 let-7a-3/7b	let-7
let-7b		↓		↑											↓	22q13.31	let-7a-3/7b	
let-7c		↓		↑									↓		↓	21q21.1	miR-99a/let-7c	
miR-99a									↓	↓		↓			↓	21q21.1	miR-99a/let-7c	miR-99
let-7i				↑											↑	12q14.1	-	let-7
miR-25					↑					↑	↑			↑	↑	7q22.1	miR-25/93/106b	miR-25
miR-93				↑					↑	↑			↑		↑	7q22.1	miR-25/93/106b	miR-17
miR-106b									↑				↑		↑	7q22.1	miR-25/93/106b	
miR-18a	↑					↑			↑	↑					↑	13q31.3	miR-17/18a/19a/20a/19b-1/92a-1	
miR-92a			↓						↑			↓			↑	13q31.3 Xq26.2	miR-17/18a/19a/20a/19b-1/92a-1 miR-106a/18b/20b/19b-2/92a-2/363	miR-25
miR-143		↓									↓				↓	5q32	miR-143/145	miR-143
miR-145		↓			↓		↓				↓			↓	↓	5q32	miR-143/145	miR-145
miR-15a				↑			↑		↑		↓				↑	13q14.2	miR-15a/16-1	miR-15
miR-15b															↑	3q25.33	miR-15b/16-2	
miR-191														↑	↑	3p21.31	miR-191/425	miR-191
miR-425													↑		↑	3p21.31	miR-191/425	miR-425
miR-221		↑	↑	↑	↑	↑					↑	↑	↑		↑	Xp11.3	miR-221/222	miR-221
miR-222			↑		↑			↑		↑	↑	↑	↑	↑	↑	Xp11.3	miR-221/222	
miR-29b															↑	7q32.3 1q32.2	miR-29b-1/29a miR-29b-2/29c	miR-29
miR-140-3P															↑	16q22.1	miR-17/18a/19a/20a/19b-1/92a-1	miR-140
miR-181a		↓													↑	9q33.3 1q32.1	miR-181a-2/181b-2 miR-181a-1/181b-1	miR-181
miR-193b															↑	16p13.12	miR-193/365-1	miR-193
miR-199a	↓	↓	↓			↓				↓	↓	↓		↓	↓	19p13.2 1q24.3	- miR-199a-2/214/3120	miR-199
miR-212															↑	17p13.3	miR-212/132	miR-132
miR-215				↑						↓					↑	1q41	miR-194-1/215	miR-192
miR-224	↑				↑			↑		↑	↑		↑	↑	↑	Xq28	miR-224-452	miR-224
miR-331														↑	↑	12q22	miR-331/3685	miR-331
miR-371-5P															↑	19q13.42	miR-371/372/373	miR-290
miR-423-5P															↑	17q11.2	miR-423/3184	miR-423
miR-423-3P															↑	17q11.2	miR-423/3184	miR-423
miR-451															↓	17q11.2	miR-144/451	miR-451
miR-487b															↑	14q32.31	miR-376c/376a-2/654/376b/376a-1/300/1185-1/1185-2 /381/487b/539/889/544/655/487a/382/134/668/485/323b	miR-154
miR-494															↑	14q32.31	miR-379/411/299/380/1197/323/758/329-1/329-2 /494/1193/543/495	
miR-130b						↑									↑	22q11.21	miR-130b/301b	miR-130
miR-130a		↓							↓		↑	↓			↓	11q12.1	-	
miR-320b															↑	1p13.1 1q42.11	- -	miR-320
miR-320c															↑	18q11.2 18q11.2	- -	
miR-320d															↑	13q14.11 Xq27.1	- -	
miR-1274A															↑	5p13.1	-	miR-1274
miR-1274B															↑	19q13.43	-	
miR-21			↑	↑	↑	↑		↑	↑			↑	↑	↑	↑	17q23.2	-	miR-21
miR-26a												↓			↓	3p22.2 12q14.1	- -	miR-26
miR-34a			↑	↑									↑		↑	1p36.22	-	miR-34
miR-122		↓	↓					↓	↓		↓	↓			↓	18q21.3	-	miR-122
miR-125b			↓				↑			↓	↓	↓		↓	↓	11q24.1 21q21.1	- -	miR-125
miR-148a				↑					↑						↑	7p15.2	-	miR-148
miR-151					↑										↑	8q24.3	-	miR-28
miR-185							↓				↓			↑	↑	22q11.21	-	miR-185
miR-210			↑							↑			↑		↑	11p15.5	-	miR-210
miR-223		↓				↓				↓		↓			↓	Xq12	-	miR-223
miR-378										↓					↓	5q32	-	miR-378
miR-638															↑	19p13.2	-	miR-638
miR-663															↑	20Centromere	-	miR-663
miR-720															↑	3q26.1	-	miR-720
miR-885-5P															↑	3p25.3	-	miR-885
miR-1246															↑	2q31.1	-	miR-1246
miR-1260															↑	14q24.3	-	miR-1260
miR-1469															↑	15q26.2	-	miR-1469
miR-1908															↑	11q12.2	-	miR-1908
miR-1909															↑	19p13.3	-	miR-1909

miR-425 up-regulation) and miR-221/222 cluster on ChrXp11.3 (miR-221 and miR-222 up-regulation).

Table 3.1 also summarized the functional miRNA family classification of these 66 significantly differentially expressed miRNAs in HCC. These 66 miRNAs belonged to a total of 46 miRNA families with slight enrichment in let-7 family (let-7a, let-7b, let-7c and let-7i), miR-17 family (miR-18a, miR-93 and miR-106b), miR-320 family (miR-320b, miR-320c and miR-320d), miR-15 family (miR-15a and miR-15b), miR-25 family (miR-25 and miR-92a), miR-130 family (miR-130a and miR-130b), miR-221 family (miR-221 and miR-222), miR-154 family (miR-487b and miR-494) and miR-1274 family (miR-1274a and miR-1274b). These data suggest that in addition of regulation at the genomic loci, the family-defining seed sequence may play a role in miRNA regulation at the processing and maturation level.

In Table 3.1, we compared our data (Thesis column or Column 15) with the 14 previous miRNA profiling studies reported in the literature (Column 2-14) and found high degree of consistency in the observed miRNA deregulation. We identified 30 novel miRNAs deregulated in HCC. Majority of these 30 miRNAs were recently discovered and not profiled in the previous studies reported in the literature. The other 36 miRNAs were all consistently reported by at least one other study except miR-181a. This gave us confidence that our method of profiling for miRNA deregulation in HCC is reliable and accurate, independent of the patient samples selected. To understand the functional relevance of these miRNA deregulations in HCC, we subsequently validated and functionally characterized for let-7a down-regulation and miR-224 up-regulation in HCC.

3.2 Characterization of let-7a down-regulation in HCC tumors

** This section is adapted from original research article,* ***Wang, Y****., Lu, Y., Toh, S. T., Sung, W. K., Tan, P., Chow, P., Chung, A. Y., Jooi, L. L. and Lee, C. G. (2010). Lethal-7 is down-regulated by the hepatitis B virus x protein and targets signal transducer and activator of transcription 3. J Hepatol 53, 57-66.*

3.2.1 Let-7a is down-regulated in tumors of HCC

As shown by the miRNA microarray profiling data in Figure 3.1 (yellow box), members of let-7 family of miRNAs, namely let-7a, let-7b and let-7c, were significantly down-regulated in tumors of HCC. Let-7 is the first miRNA identified in humans and evolutionarily conserved from *C. elegans* to humans. Let-7 was demonstrated to play crucial roles in developmental processes. Its reduced expression has been reported in a number of human cancers[138]. Hence, we would like to further characterize the functional relevance of let-7 down-regulation in human HCC.

We proceeded to validate the miRNA microarray findings with Taqman reverse transcription quantitative PCR (RT-qPCR) using specific human let-7a Taqman miRNA assays in 20 normal liver samples as well as paired tumor and adjacent non-tumor samples from 20 HBV-associated HCC patients. As shown in Figure 3.2.1, let-7a expression is significantly lower in tumors compared to the adjacent non-tumorous liver tissues of HCC patients ($p<0.001$). This is consistent with our observation of let-7 down-regulation in HCC tumors from the miRNA microarray data. Additionally, both HCC tumor and adjacent non-tumorous liver tissues showed significantly lower let-7a expression compared to normal liver tissues ($p<0.001$ and $p<0.01$, respectively)

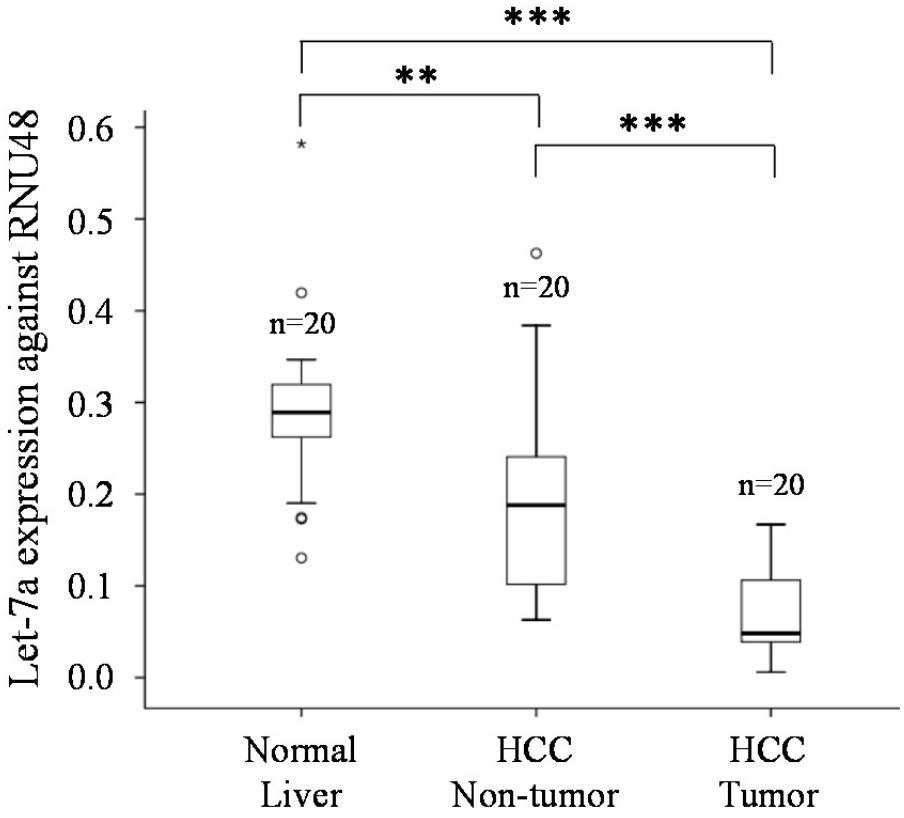


Figure 3.2.1 Let-7a expression is significantly lower in HCC tumor than non-tumor samples. Box plot of let-7a expression in the normal liver samples from 20 metastatic colorectal cancer patients (Normal Liver), the adjacent non-tumorous liver (HCC Non-tumor) and tumor tissues (HCC Tumor) from 20 HBV-associated HCC patients. ** denotes $P<0.01$ and *** denotes $P<0.001$.

(Published data)

As the 20 HCC patients examined in let-7a validation are all HBV positive and HBV X protein (HBx) is known to be selectively over-expressed in tumors of HCC, we proceeded to evaluate whether reduced let-7a expression in these HCC patients were related to HBx expression.

3.2.2 HBx expression is inversely correlated with let-7a expression in hepatocellular carcinoma patients

We proceeded to examine the HBx protein expression in HCC patient samples through Western blot analysis with a custom-made polyclonal rabbit-anti-HBx antibody against full length HBx as antigen. As shown by Figure 3.2.2 (1), HBx protein is detectable in all 20 HBV-associated HCC cases. In 16 of these 20 HBV^+ HCC patients, HBx is selectively over-expressed in the tumor tissues compared to the paired adjacent non-tumor tissues while 4 HCC patients showed higher HBx expression in non-tumor than tumor tissues. We subsequently examined the correlation between HBx and all members of let-7 miRNAs using Pearson correlation and observed statistically significant inverse correlation between HBx and let-7a ($p<0.01$), let-7b ($p<0.05$) & let-7c ($p<0.01$) (Figure 3.2.2 (2)). Furthermore, when the HBV^+ HCC patients were sub-grouped based on the relative HBx status between tumor and non-tumor tissues as shown in Figure 3.2.2.(3), let-7a expression is significantly lower while HBx expression is significantly higher in the tumor compared to the non-tumorous liver tissues in 16 HCC patients ($p<0.001$). In contrast, although not statistically significant, let-7a expression is higher while HBx expression is lower in the tumor compared to the non-tumorous tissues in the remaining four HCC patients. Taken together, our data strongly suggests that HBx may play a role in the observed let-7 down-regulation in HCC tumors.

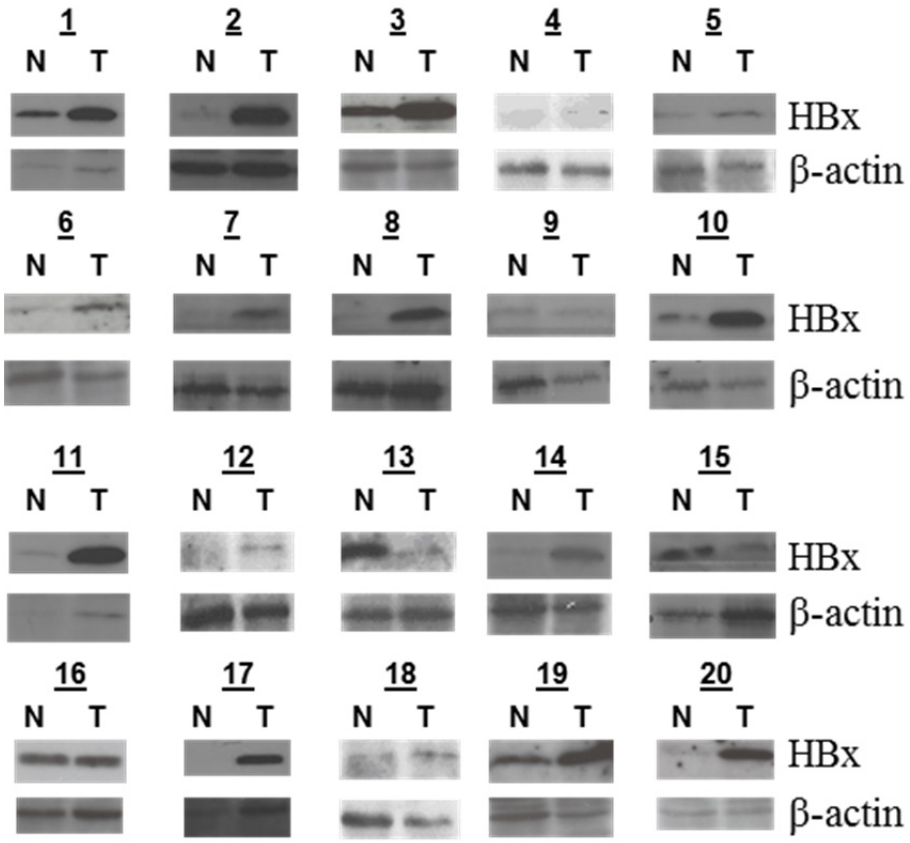


Figure 3.2.2. (1) HBx is selectively over-expressed in tumors of HCC
Western blot detection of HBx protein in tumor (T) and paired adjacent non-tumor (N) samples from 20 HBV-associated HCC patients, normalized against β-actin as endogeneous control.

(Published data)

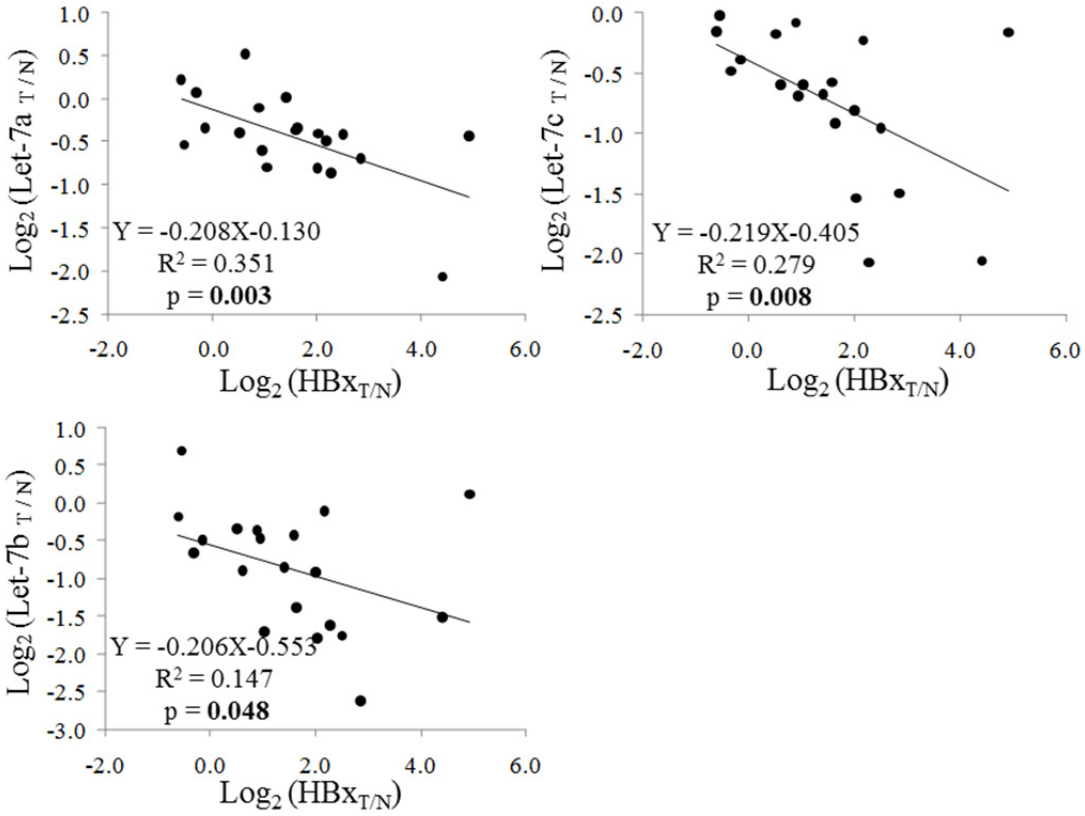


Figure 3.2.2.(2) HBx expression is inversely correlated with let-7a, let-7b and let-7c expression in HCC patients. Scatter plots showing the statistically significant correlation in the relative expression of HBx and the relative expression of let-7a (**p<0.01**), let 7b (**p<0.05**) and let-7c (**p<0.01**) in the tumor versus paired adjacent non-tumorous tissues of 20 HCC patients. Each spot represents data from one HCC patient presented in the Log_2 scale and the linear regression line is depicted as the solid line.

(Published data)

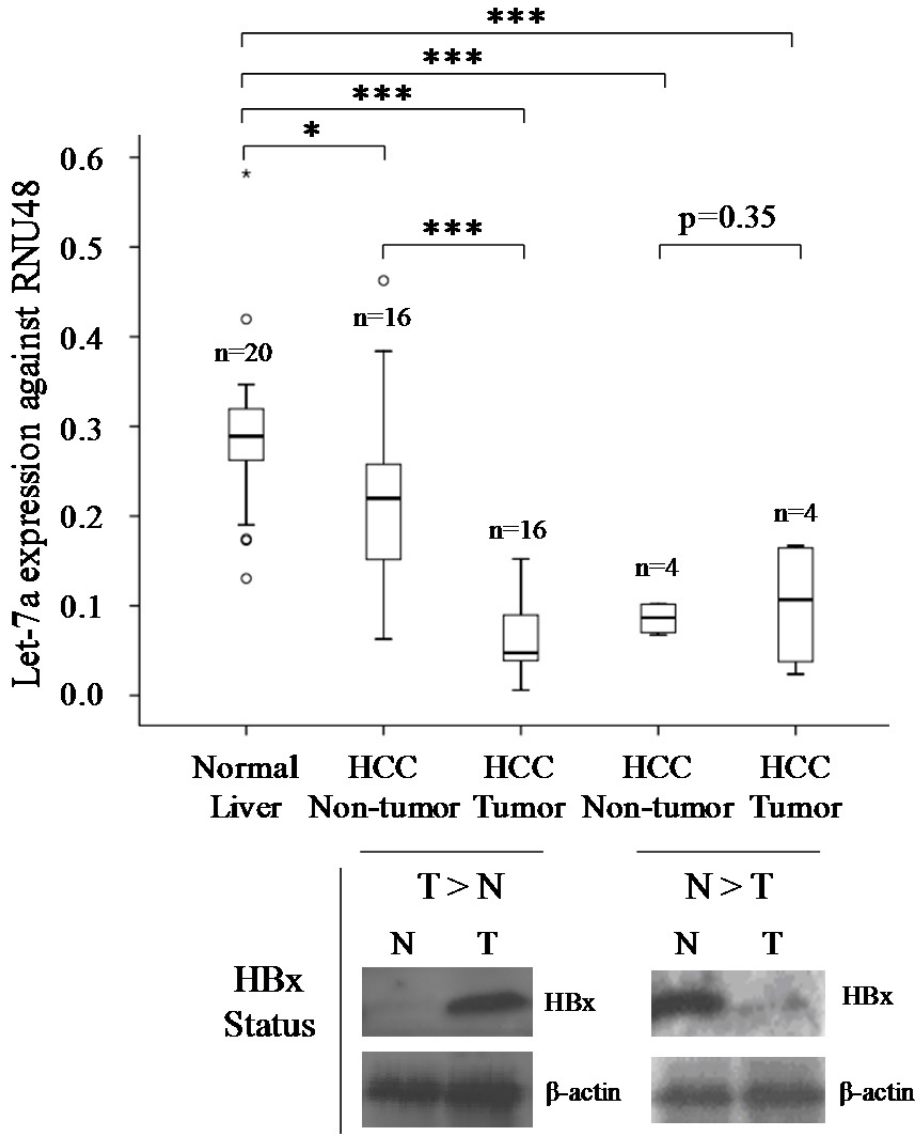


Figure 3.2.2. (3) Let-7a expression is only significantly reduced in HCC tumors where HBx is selectively over-expressed, compared to the paired adjacent non-tumor tissues. Box plot of let-7a expression in 20 normal liver samples (Normal Liver), 16 HCC patients with higher HBx protein expression in tumor than paired non-tumorous tissues and 4 HCC patients with lower HBx protein expression in tumor than paired non-tumorous tissues, measured with Western Blot analysis. Representative western blot of HBx versus β-actin as loading control are shown below the box plot to illustrate the HBx status between the two groups of HCC patients. * denotes $p<0.05$ and *** denotes $P<0.001$.

(Published data)

3.2.3 Let-7 family of miRNAs are significantly down-regulated in HBx-expressing HepG2 cells

To ascertain whether HBx directly deregulates the expression of cellular miRNAs such as let-7, we transiently expressed HBx in HepG2 cells through a recombinant adenovirus carrying full length HBx. The expression profile of 286 human miRNAs was examined in HBx-expressing versus control HepG2 cells from three independent experiments. The miRNA microarray data can be obtained from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=tlmdlkmyimcewhm&acc=GSE17474, Accession number: GSE17474.

Ninety miRNAs were found to exhibit significant expression in all three paired biological samples and these miRNAs were presented in Figure 3.2.3.(1)A as a Volcano plot. In the volcano plot, the X-axis is the logarithmic transformation to the base 2 of the mean fold change of miRNA expression in HBx versus Control HepG2 cells and the Y-axis is the negative logarithm to the base 10 of the p-value of Student's t test. Significantly differentially expressed miRNAs were identified as absolute mean fold change of greater than 1.5 and p-value of less than 0.01 and located in the two upper lateral quandrants. Seven miRNAs (miR-30c, miR-193b, miR-342, miR-199a, miR-125a, miR-99b and miR-191) were found to be significantly up-regulated while 11 miRNAs (miR-196a, miR-106a, miR-20a and let-7 family of miRNAs) were found to be significantly down-regulated in HBx-expressing HepG2 cells compared to the control cells (Figure 3.2.3.(1)B). Notably, all members of the let-7 family were down-regulated, eight of which showed statistical significance ($p<0.05$). (Table 3.2.3).

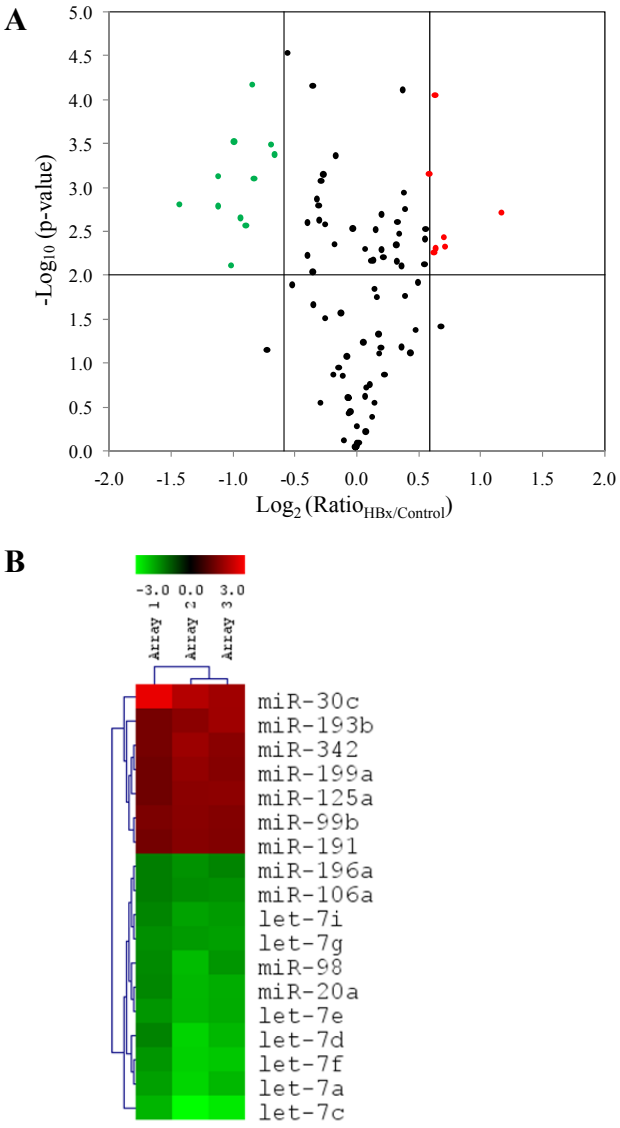


Figure 3.2.3. (1) Profile of miRNAs deregulated by HBx in HepG2 cells. **(A)** Volcano plot showing 90 miRNAs that were significantly expressed in HBx cells. miRNA microarray data were collected by comparing the miRNA expression in HBx-expressing HepG2 cells (HBx cells) versus the Control cells from three biological replicates. Y-axis: -log10 (p-value); X-axis: log2(Ratio(HBx/Control)). Differential expression of miRNAs in HBx cells versus Control cells that were both biologically and statistically significant were indicated in the two upper-lateral quadrant with absolute fold change > 1.5 and p-value < 0.01. Green spots indicated miRNAs that were significantly down-regulated while red spots indicated miRNAs that were significantly over-expressed in HBx cells. **(B)** Hierarchical clustering of miRNAs that were differentially expressed in HBx-expressing cells using Euclidean correlation with average linkage. Up-regulated miRNAs are represented by Red blocks while down-regulated miRNAs are shaded Green.

(Published data)

Figure 3.2.3.(1)B presented the 18 significantly differentially expressed miRNAs in a Heat Map using unsupervised hierarchical clustering with Euclidean distance and average linkage analysis. We chose to validate the expression of two up-regulated miRNAs (miR-30c and miR-199a) and all the members of the let-7 family using reverse transcription Taqman real-time PCR to confirm the miRNA microarray findings. The data was normalized against endogenous control RNU48. The let-7 family of miRNAs showed greatly variable endogenous level of expression. Expression of let-7a, let-7b and let-7c were high whereas the expression of let-7e and let-7i were very low (Figure 3.2.3.(2)A). Nonetheless, all 9 mature miRNAs were validated to be significantly down-regulated in HBx-expressing HepG2 cells, compared to those of the Control HepG2 cells. (Figure 3.2.3.(2)A and Table 3.2.3). To ensure that the observed effect of HBx on let-7 is not cell line specific, we repeated the experiment in a second liver cell line SNU-182 [253]. As evident in Figure 3.2.3.(2)B, expression of let-7a, let-7b, let-7c, let-7e and let-7i were significantly reduced in HBx-expressing SNU-182 cells. Taken together, we have demonstrated a distinct miRNA expression profile, including the down-regulation of the entire let-7 family of miRNAs, which was associated with HBx in HepG2 cells. The down-regulation of selected members of the let-7 miRNAs by HBx is reproducible in SNU-182 cells.

Hence, we have demonstrated that HBx plays a role in down-regulating the expression of let-7a, let-7b and let-7c in liver cancer cell lines and is inversely correlated with these let-7 miRNAs in HCC patient samples. This suggests that the down-regulation of let-7 miRNAs may happen as an

early event in HCC development upon HBV infection. The down-regulation of let-7 may thus play an important role in hepatocellular carcinogenesis.

3.2.4 Let-7 negatively regulates cell proliferation

Let-7 miRNAs have been reported to play important roles in cell differentiation and cell proliferation (See review [254]). To evaluate the functional significance of HBx-mediated let-7 down-regulation, levels of cellular let-7 expression was altered by exogenously introducing the let-7 precursor or let-7 inhibitor into either HepG2 or SNU-182 cells. As the members of the let-7 family of miRNAs have previously been shown to have similar functions [255], let-7a was selected as a representative member of the let-7 family of miRNAs since it is one of the most highly expressed let-7 miRNA in liver cells and consistently down-regulated by HBx in both HepG2 and SNU-182 cells (Figure 3.2.3.(2)). A significant 30% decrease ($p<0.001$) in cell proliferation (Figure 3.2.4B, left panel) was observed when let-7a precursor was introduced into HepG2 cells (Figure 3.2.4A, left panel). Inversely, when let-7a expression was inhibited by ~50% through the introduction of let-7a inhibitor (Figure 3.2.4A, left panel), there was a corresponding significant increase ($P<0.01$) in cell proliferation (Figure 3.2.4B, left panel) in HepG2 cells. The expression of unrelated miR-100 which was used as a control remained undisturbed, demonstrating the specificity of the small oligos in perturbing endogeneous let-7a level. Similar results were observed in the SNU-182 cells (Figure 3.2.4 A&B, right panel). Our data confirms the observation made by Johnson *et al* previously [255] highlighting the role of let-7 in negatively regulating cell proliferation in HepG2 cells.

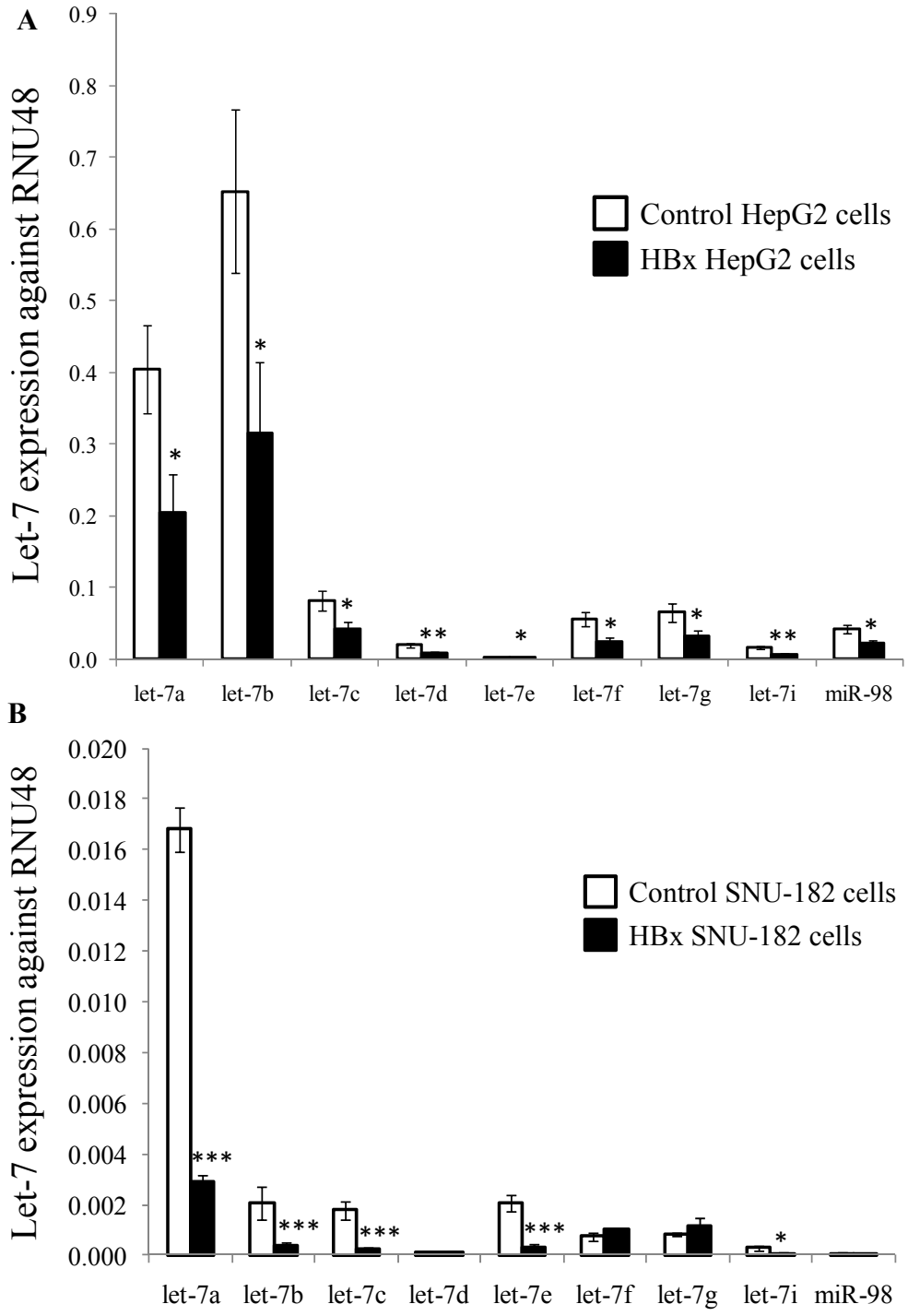


Figure 3.2.3.(2) Validation of let-7 down-regulation in HepG2 and SNU-182 cells. Let-7 expression normalized against RNU48 measured using reverse transcription Taqman real-time PCR in HBx expression HepG2 cells versus Control cells **(A)** and HBx-expressing SNU-182 cells versus Control cells **(B)**. Data is presented as Mean ± S.E. from three independent experiments. * denotes $p<0.05$, ** denotes $P<0.01$ and *** denotes $P<0.001$.

(Published data)

Table 3.2.3 Significantly differentially expressed miRNAs in HBx-expressing HepG2 cells versus Control cells as measured with miRNA microarray and validated with Taqman RT-qPCR

miRNA	Mean Fold Change (microarray)	p-value	Mean Fold Change (Taqman)	p-value
miR-30c	+2.24	1.90E-03 **	+2.32	3.54E-02 *
miR-193b	+1.63	4.59E-03 **	+1.78	1.31E-01
miR-199a	+1.55	5.00E-03 **	+3.41	4.33E-02 *
miR-191	+1.50	6.85E-04 ***	+4.61	9.99E-02
let-7a	-2.18	7.33E-04 ***	-1.98	2.54E-02 *
let-7b	-1.65	7.08E-02	-2.07	3.38E-02 *
let-7c	-2.70	1.53E-03 **	-1.95	3.48E-02 *
let-7d	-2.03	7.61E-03 **	-2.33	7.33E-03 **
let-7e	-1.98	3.03E-04 ***	-2.25	2.33E-02 *
let-7f	-2.18	1.63E-03 **	-2.27	1.62E-02 *
let-7g	-1.80	6.53E-05 ***	-2.04	3.05E-02 *
let-7i	-1.77	7.67E-04 ***	-2.28	8.90E-03 **
miR-98	-1.85	2.64E-03 **	-1.90	1.65E-02 *

Note: * denotes $p<0.05$, ** denotes $P<0.01$ and *** denotes $P<0.001$.

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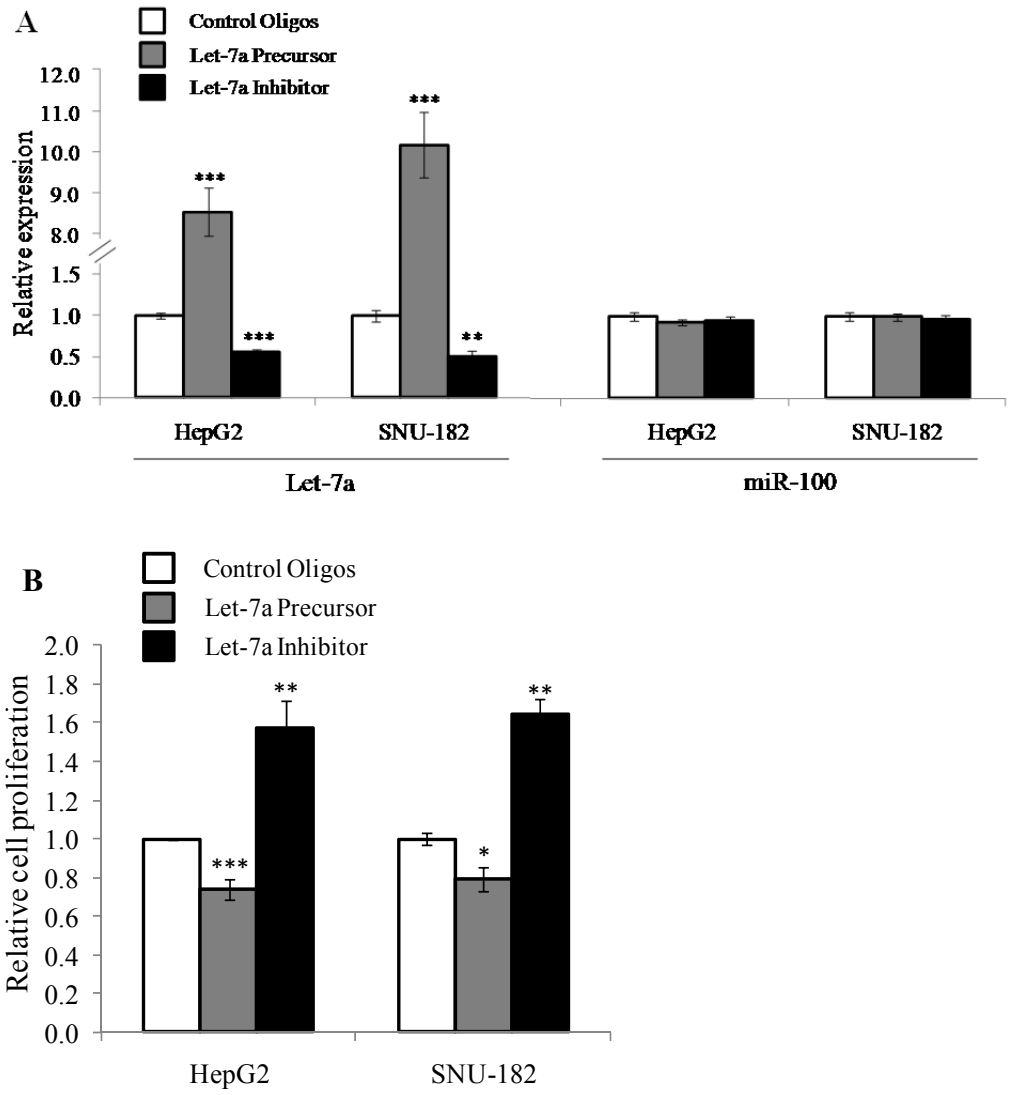

Figure 3.2.4 Let-7 negatively regulates cell proliferation. A. Relative let-7a (left two panels) and miR-100 (right two panels) expression normalized against RNU48, measured using reverse transcription Taqman real-time PCR, in HepG2 and SNU-182 cells transfected with Control oligos, let-7a precursor or let-7a inhibitor. **B.** Relative cell proliferation measured using the WST1 assay in the same experimental setup. Data presented as Mean ± SE from three independent experiments. * denotes $P<0.05$, ** denotes $P<0.01$ and *** denotes $P<0.001$.

(Published data)

3.2.5 STAT3 is a direct cellular target of let-7

3.2.5.(1) Let-7a specifically interacts with the putative let-7a binding site along STAT3 3'UTR to negatively regulate reporter gene expression.

To elucidate the cellular targets through which let-7 affects cell proliferation, we performed *in silico* analysis with miRecords which is an online resource that compiles predicted miRNA targets produced by 11 established miRNA target prediction programs [256]. Six of these 11 prediction programs, namely miRanda, NBmiRTar, PicTar, PITA, RNAHybrid and RNA22, predicted Signal Transducer and Activator of Transcription 3 (STAT3) as a putative let-7 target. STAT3 is the central mediator of JAK/STAT pathway that plays an important role in cell proliferation. Several studies have previously demonstrated the role of HBx in activating STAT3 [257, 258]. Hence we proceeded to investigate whether HBx can also regulate STAT3 expression through down-regulating let-7 miRNAs. One strong putative let-7 binding site was identified along STAT3 3'UTR by miRanda (Release 2008). To validate whether let-7 directly targets STAT3, we cloned the wild-type 3'UTR of STAT3 as well as a mutant 3'UTR which is mutated at the putative let-7 binding site downstream the β-galactosidase (β-gal) reporter gene (Figure 3.2.5.(1)A). As evident in Figure 3.2.5.(1)B, introduction of let-7a precursors into cells containing wild-type STAT3 3'UTR reporter construct resulted in significantly lower ($P<0.001$) β-gal activity compared to cells carrying the mutant STAT3 3'UTR reporter construct in which the let-7 binding site is mutated. Conversely, when let-7a inhibitor was introduced, cell containing the wildtype STAT3 3'UTR reporter construct showed significantly higher ($p<0.01$) β-gal activity compared to cells

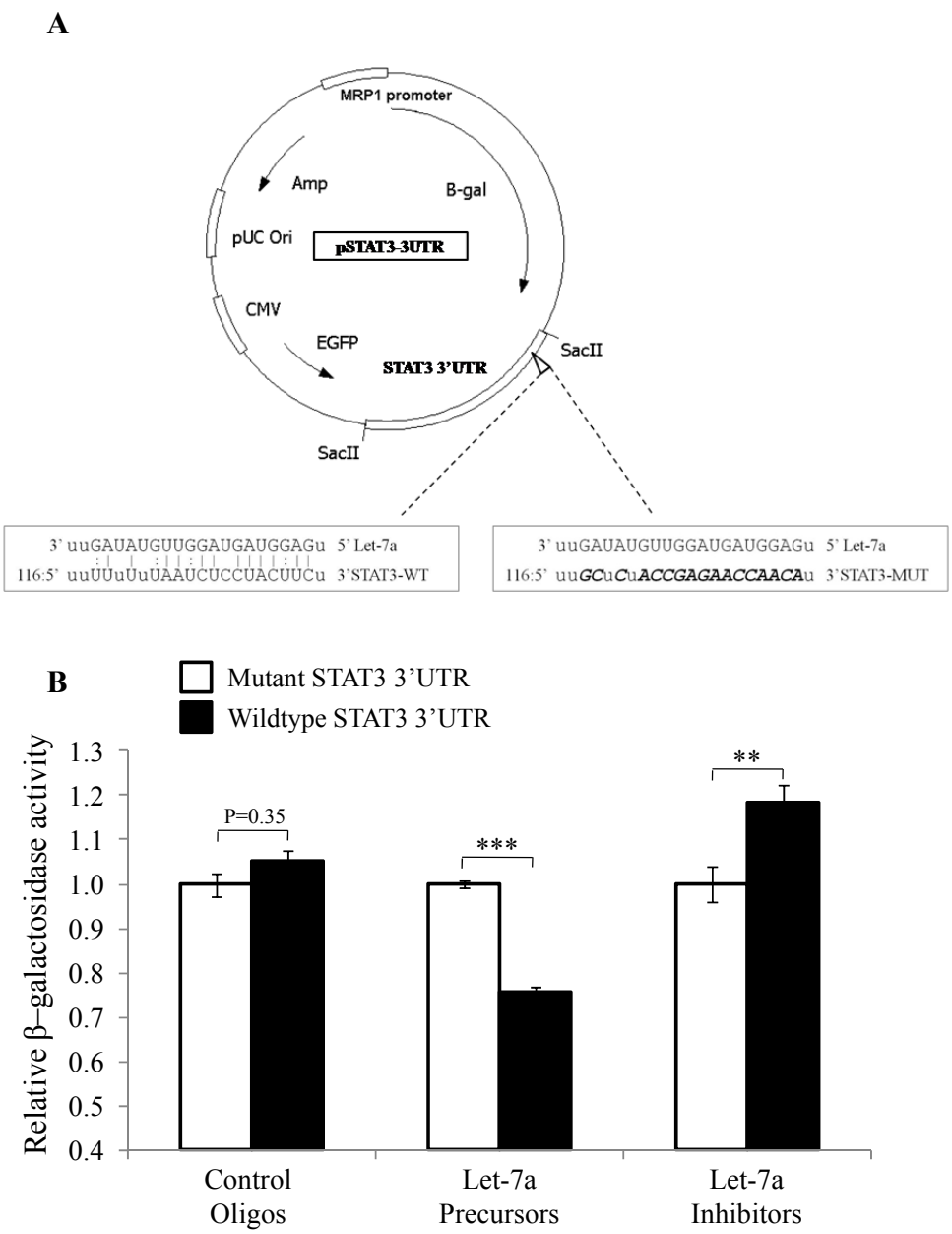

Figure 3.2.5.(1) Let-7a interacts with the putative binding sites on STAT3 3'UTR. (A) Schematic diagram of the constructs utilized to validate that the let-7 targets the 3'UTR region of STAT3. The wildtype STAT3 3'UTR carrying the predicted let-7 binding site (left box) or the mutant STAT3 3'UTR carrying the mutated let-7 binding site (right box with the mutation residues in BOLD) were cloned downstream β-galactosidase reporter gene driven by MRP1 promoter. **(B)** Effect of let-7a on STAT3 3'UTR examined through normalized β-galactosidase activity in cells co-transfected with Control oligos, let-7a precursor or let-7a inhibitor and wildtype STAT3 3'UTR (Black bar) or mutant STAT3 3'UTR reporter construct (White bar). ** denotes $p<0.01$ and *** denotes $p<0.001$.

(Published data)

carrying the mutant STAT3 3'UTR reporter construct. No significant difference was observed between cells carrying wild-type or mutant STAT3 3'UTR reporter construct when Control Oligos were introduced. Hence, our data demonstrate that let-7a specifically interacted with the putative let-7a binding site on STAT3 3'UTR to negatively regulate the β-gal reporter gene activity.

3.2.5.(2) Let-7a negatively regulates endogeneous STAT3 expression.

To evaluate whether let-7a affects endogenous STAT3 expression, we manipulated the endogenous let-7a expression with specific let-7a precursors or inhibitors. The endogenous STAT3 transcript and protein expression was found to be significantly decreased ($P<0.001$) when let-7a precursor was introduced (Figure 3.2.5.(2)). Conversely, when the let-7a inhibitor was introduced, the endogenous STAT3 transcript and protein expression was also significantly increased ($P<0.01$) (Figure 3.2.5.(2)). As a positive control, we concurrently examined if the addition of let-7a precursor or inhibitor into cells affected a previously reported validated cellular target of let-7a, c-Myc [76]. As evident in Figure 3.2.5.(2) (right panel), similar decrease and increase in c-myc protein expression was observed when let-7a precursor and inhibitor, respectively, was introduced into the cells. Taken together, our data demonstrated that let-7a physically interacted with the 3'UTR of STAT3 to negatively regulate STAT3 cellular expression.

3.2.5.(3) STAT3 inhibition phenocopies let-7a over-expression.

To ascertain that STAT3 is indeed a target through which let-7a affects cell proliferation, we inhibited STAT3 expression with siRNA that specifically

targets STAT3 (si-STAT3) and examined its effect on cell proliferation. As shown in Figure 3.2.5.(3)B, STAT3 inhibition reduced cell proliferation (rightmost lane), phenocopying the effect of overexpression of let-7a (middle lane). Figure 3.2.5.(3)A shows the expression of let-7a and STAT3 when let-7a precursor and si-STAT3 were introduced into the cells. Moreover, STAT3 expression was also found to positively correlate with HBx expression in HCC patients (Figure 3.2.5.(4)). Taken together, our data demonstrated that let-7a regulates cell proliferation at least partially through its direct target, STAT3.

3.2.6 Down-regulation of Let-7 by HBx supports cell proliferation in HBx-expressing cells

To reaffirm the relationship between let-7a and STAT3 in HBx-expressing cells, we examined the expression of let-7a and STAT3 in HepG2 cells transduced with Control or HBx recombinant adenoviruses. As evident in Figure 3.2.6.(1), let-7a showed significantly reduced expression ($p<0.05$) while STAT3 expression was significantly increased at both the transcript ($p<0.01$) and protein ($p<0.05$) levels in HBx-expressing cells versus Control cells. As HBx down-regulated let-7 and let-7 negatively regulates cell proliferation, we hypothesized that HBx would upregulate cell proliferation through down-regulating let-7a. Interestingly, cell proliferation is not significantly different in control- versus HBx-infected cells (Figure 3.2.6.(2), leftmost panel, 1st two bars). Nonetheless, when let-7a precursors were introduced, both let-7a as well as STAT3 expression in the HBx cells was restored to a similar level as those in the control cells (Figure 3.2.6.(2), middle and rightmost panels). Introduction of let-7a also results in the significant inhibition of cell proliferation in both control- and HBx-expressing cells

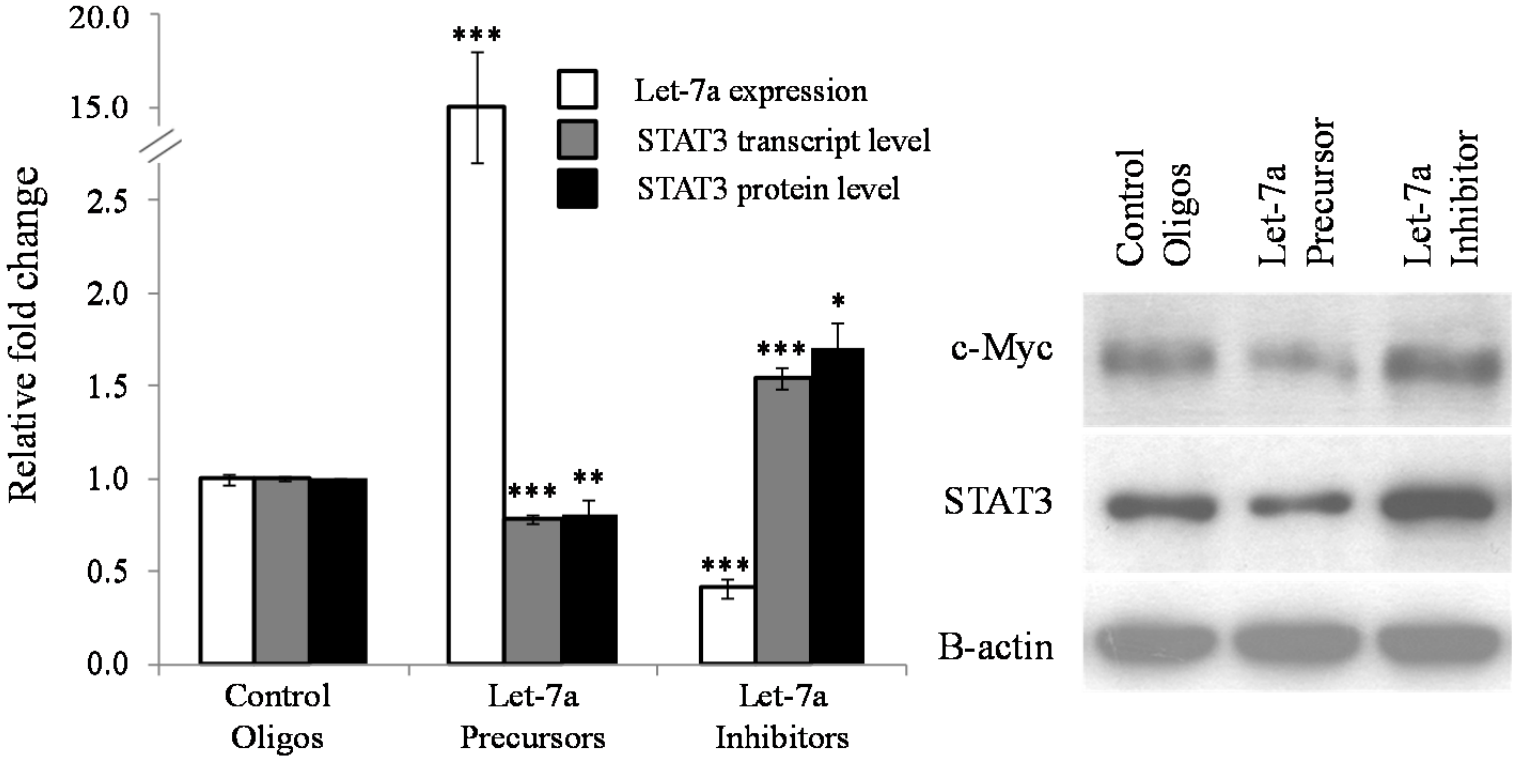

Figure 3.2.5.(2) Let-7a negatively regulates endogeneous STAT3 expression. *Left Panel*: Graph depicting relative fold change of let-7a expression (normalized against RNU48) (white bar), STAT3 transcript (grey bar) and protein (black bar) expression (normalized against β-actin) in HepG2 cells transfected with Control oligos, let-7a precursor or let-7a inhibitor. Right Panel: Representative western blot showing protein expression of c-Myc, STAT3 against β-actin in cells transfected with Control oligos, let-7a precursor or let-7a inhibitor. Data presented as Mean ± S.E. from three independent experiments. * denotes $P<0.05$, ** denotes $P<0.01$ and *** denotes $P<0.001$.

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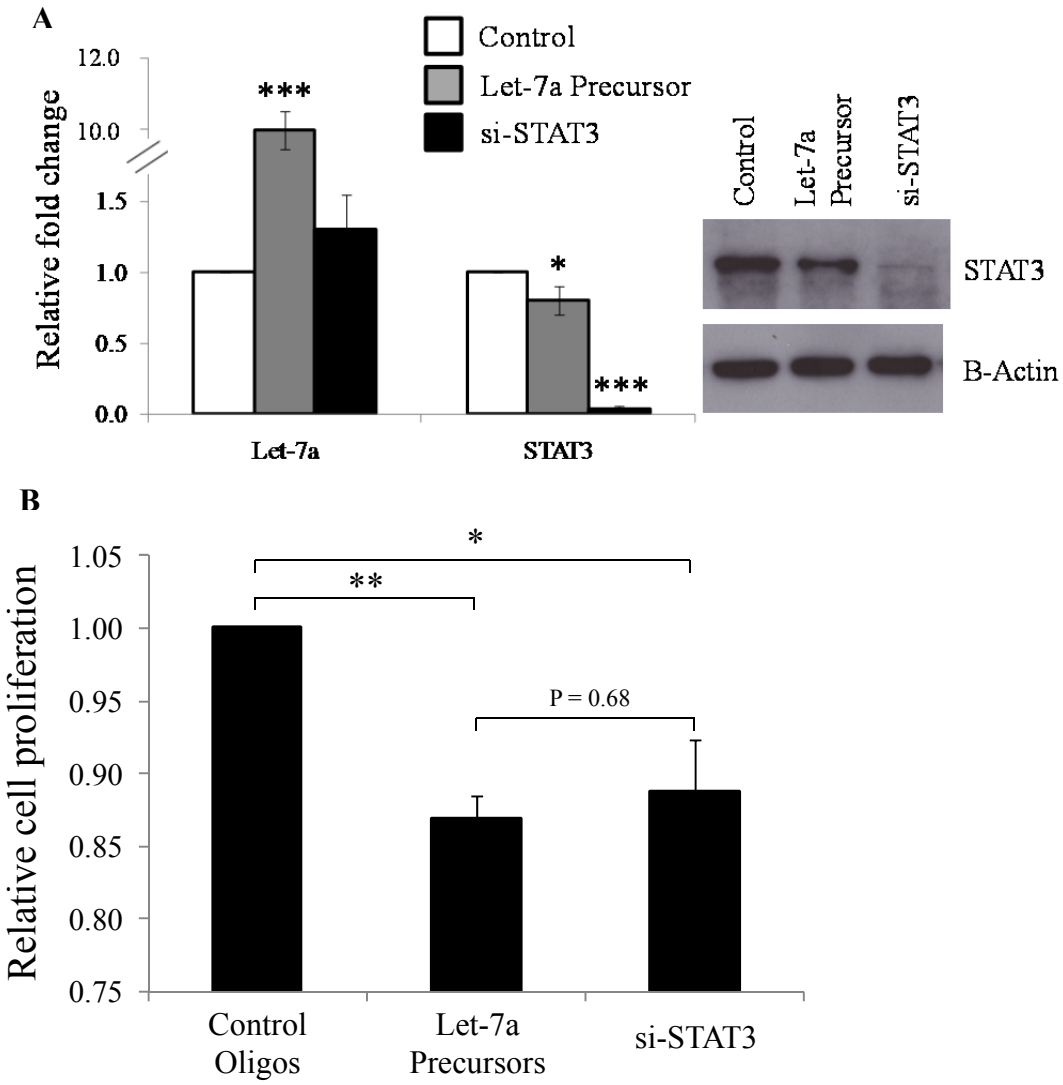

Figure 3.2.5.(3) Cells whose STAT3 expression is inhibited shows similar proliferation phenotype as cells over-expressing let-7a. (A) Expression of let-7a and STAT3 after the introduction of either let-7a or siRNA against STAT3, measured with RT-qPCR (left panel) and Western blot analysis (right panel). **(B)** Relative cell proliferation of HepG2 cells transfected with Control oligos, let-7a precursor or siRNA against STAT3 measured using the WST1 assay. * denotes $P<0.05$, ** denotes $P<0.01$ and *** denotes $P<0.001$.

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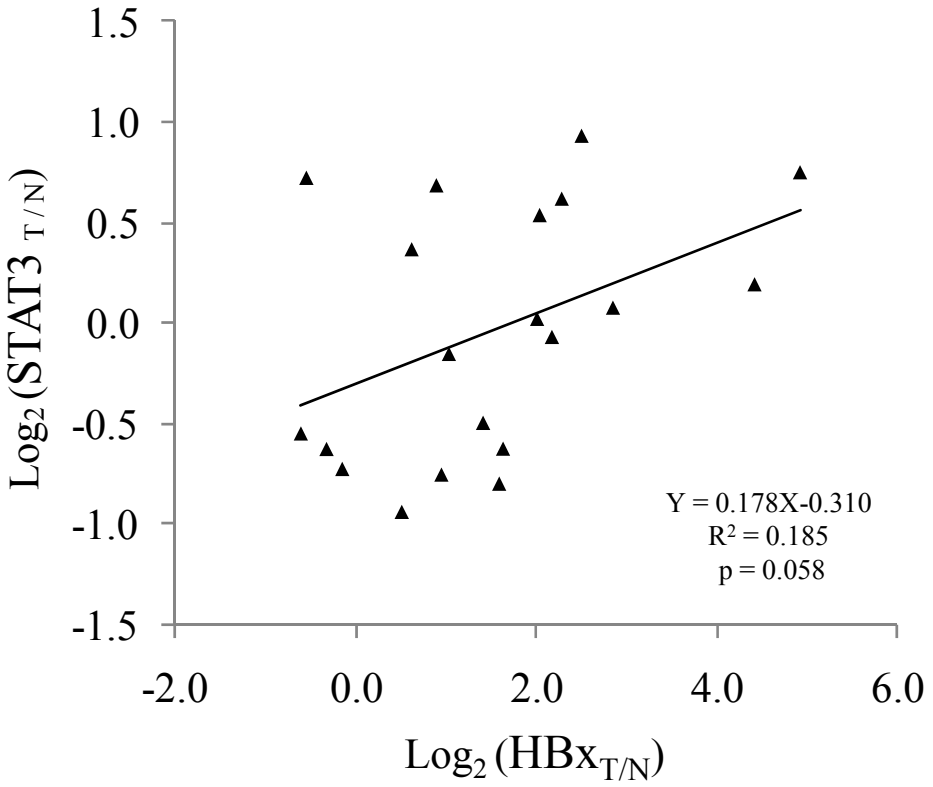

Figure 3.2.5.(4) HBx expression is postively correlated STAT3 expression in HCC patients. Scatter plots showing the positive correlation in the relative expression of HBx and the relative expression of STAT3 (p=0.058) in the tumor versus paired adjacent non-tumorous tissues of 20 HCC patients. Each spot represents data from one HCC patient presented in the Log_2 scale and the linear regression line is depicted as the solid line.

(Published data)

(Figure 3.2.6.(2), leftmost panel) with the reduction of cell proliferation in HBx-expressing cells being twice as much as that observed in the control cells ($p<0.05$). As HBx has long been known as a pleiotropic protein implicated in both cell death [251] and cell proliferation [259], our data indirectly suggests that HBx's effect on cell apoptosis may negate its effect on cell proliferation.

To reaffirm that HBx's role in cell proliferation is through its down-regulation of let-7a, we limited the effect of HBx on apoptosis by treating the cells with a general apoptosis inhibitor, zVAD, before assaying for cell proliferation. As shown by Figure 3.2.6.(3), when apoptosis was not inhibited, HBx-expressing cells showed similar cell proliferation despite ~50% more cell death, compared to the control cells. HBx-expressing cells only showed significantly higher cell proliferation (~30%, $p<0.01$) than the control cells upon treatment with apoptosis inhibitor. The treatment with control or apoptosis inhibitor did not affect HBx's ability to down-regulate let-7a and up-regulate STAT3 (Figure 3.2.6.(3)). The above observations thus provided direct evidence that the pleiotropic HBx can influence both cell death and cell proliferation and HBx-mediated down-regulation of let-7 may function to support cell proliferation in HBx cells. Taken together, these data further confirms the pleiotropic nature of HBx protein and demonstrates that HBx-mediated down-regulation of let-7 and up-regulation of STAT3 plays a role in maintaining cell proliferation to counter-balance its effect on cell apoptosis.

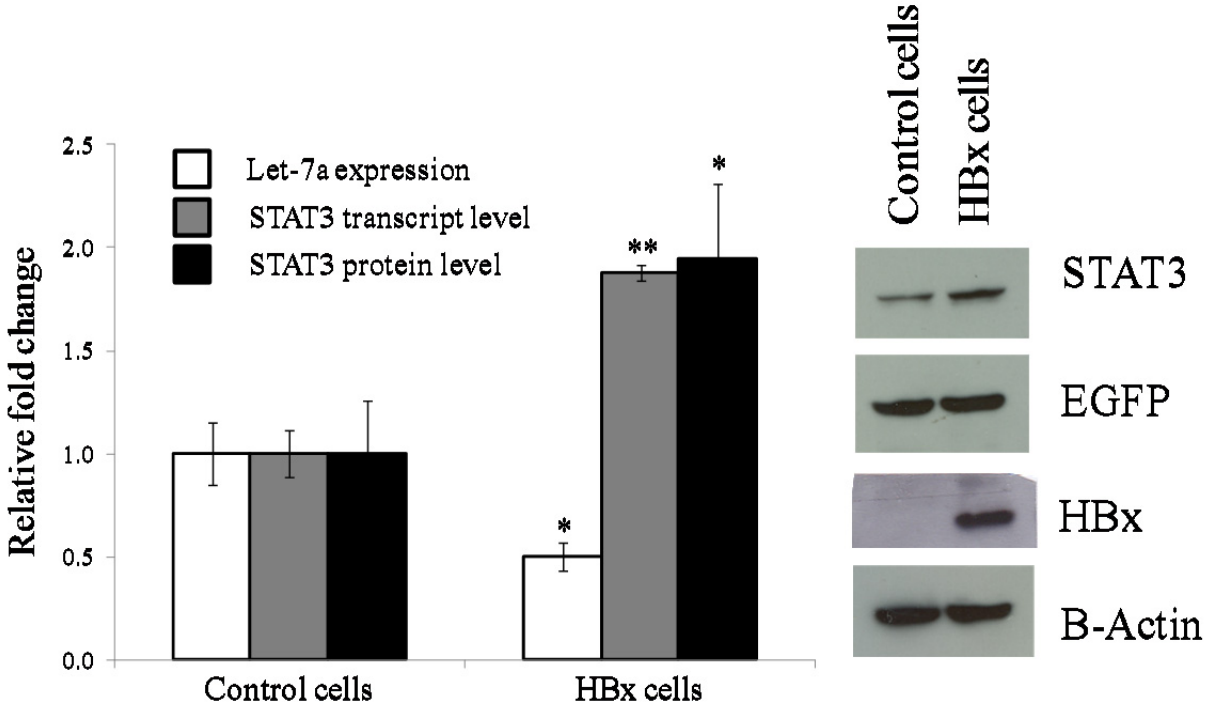

Figure 3.2.6.(1) HBx-expressing cells showed reduced let-7a and increased STAT3 expression. Left panel: Relative expression of let-7a (white bar), STAT3 transcript (grey bar) and protein (black bar) in HBx-expressing HepG2 cells versus Control cells. Right panel: Representative western blotting showing protein expression of STAT3, EGFP and HBx against β-actin.

(Published data)

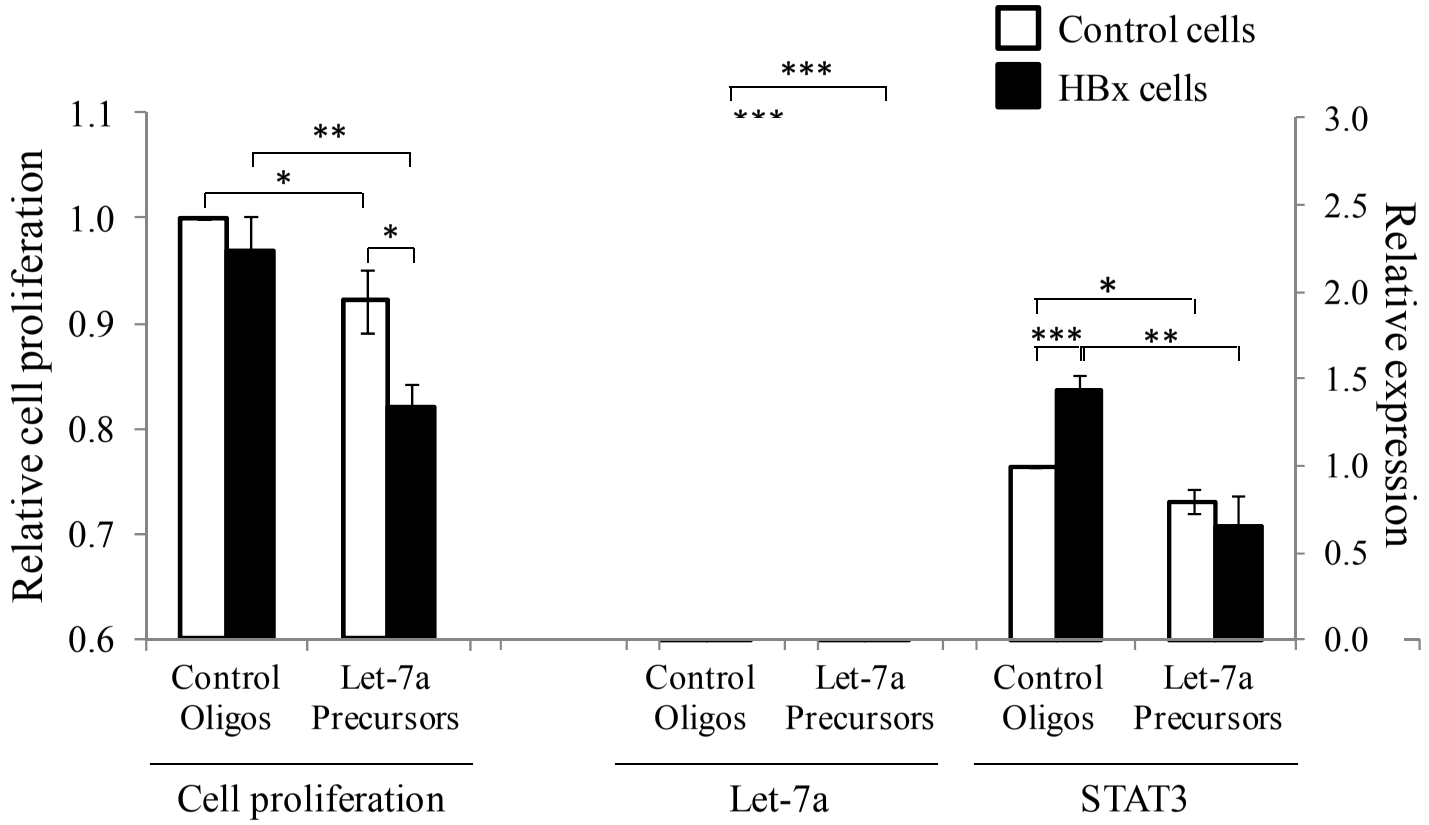

Figure 3.2.6.(2) HBx-expressing cells showed greater cell proliferation inhibition when let-7 down-regulation was rescued. Relative cell proliferation measured with WST-1 assay (Leftmost panel) and relative expression of let-7a and STAT3 (right two panels) in HBx or Control cells transfected with either Control Oligos or Let-7a precursors. Data presented are expressed as Mean ± S.E. from three independent experiments. * denotes $P<0.05$, ** denotes $P<0.01$ and *** denotes $P<0.001$.
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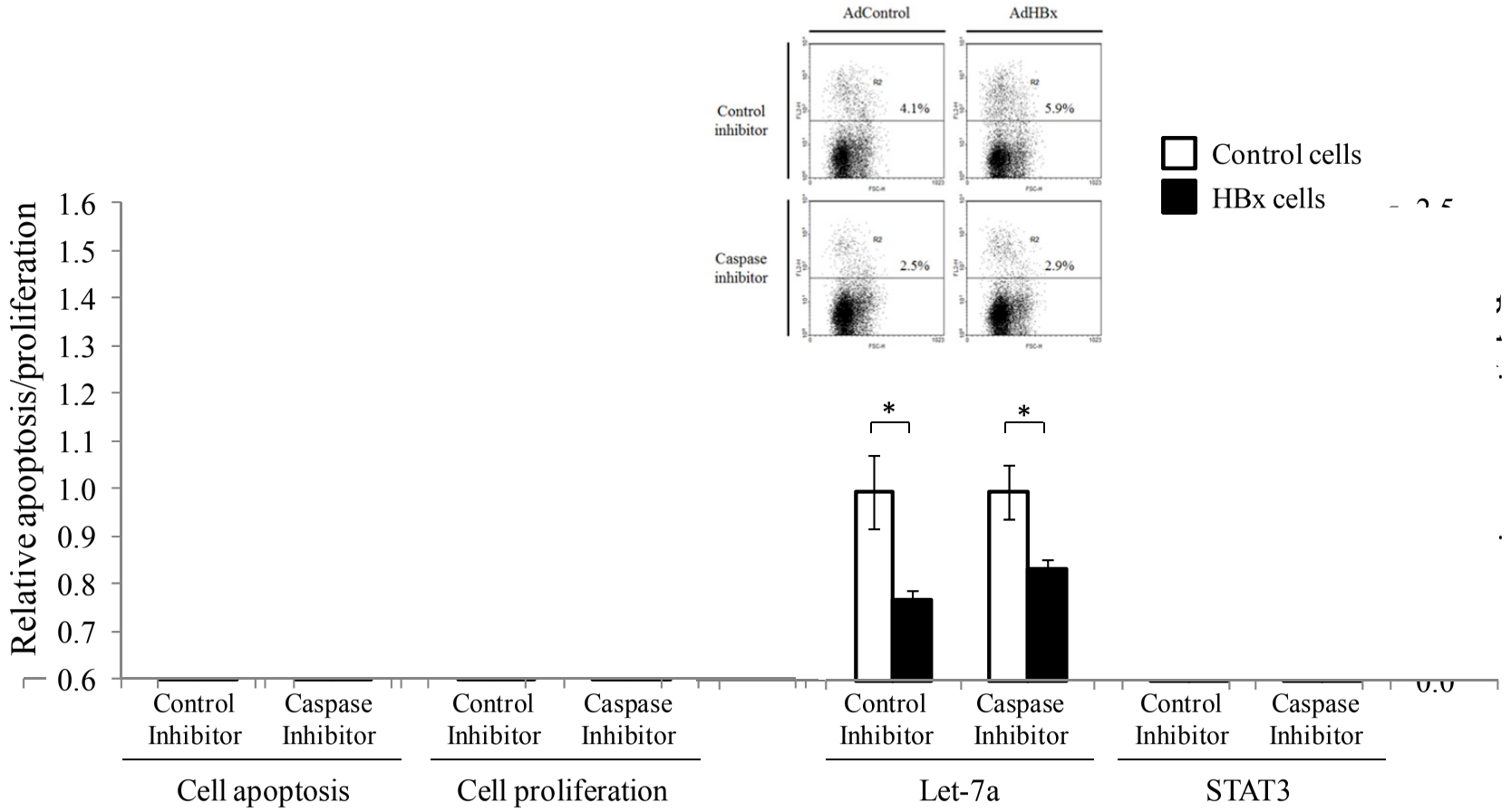

Figure 3.2.6.(3) HBx-expressing cells showed increased cell proliferation upon treatment with apoptosis inhibitor zVAD. *Left two panels:* Relative cell apoptosis measured with Annexin V assay and relative cell proliferation measured with WST-1 assay in HBx or Control cells treated with control or apoptosis inhibitor, zVAD. *Right two panels:* Corresponding let-7a and STAT3 expression under the same experimental conditions. *Center panel*: Representative FACS plot showing dead cells with high Annexin V staining. Data presented are expressed as Mean ± S.E. from three independent experiments. * denotes P<0.05, ** denotes P<0.01 and *** denotes P<0.001.
(Published data)

3.3 Characterization of miR-224 over-expression in HCC tumors

This section is adapted from original research article,* ***Wang, Y.,* *Lee, A. T., Ma, J. Z., Wang, J., Ren, J., Yang, Y., Tantoso, E., Li, K. B., Ooi, L. L., Tan, P. et al. (2008). Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. J Biol Chem, with additional unpublished data presented.*

3.3.1 miR-224 is over-expressed in tumors of HCC

We chose to further characterize miR-224 which is the most up-regulated miRNA in the tumors of HCC patients [61]. As shown in Figure 3.3.1A, miR-224 expression is significantly higher ($p<0.001$) in tumor samples compared to the adjacent non-tumor samples from HCC patients. This is further confirmed by Northern blot analysis with specific miR-224 probes on selected HCC patient samples (Figure 3.3.1B). Notably, miR-224 expression is lowest in the normal liver sample. Its expression significantly increased ($p<0.001$) in the non-tumor samples and further increased ($p<0.001$) in the tumor samples (Figure 3.3.1A). This suggests that miR-224 expression may increase as liver disease progresses, making it a possible biomarker for liver. Therefore, we proceeded to functionally characterize this miRNA to understand its role in HCC.

3.3.2 miR-224 over-expression decreases cell viability without affecting cell growth.

The functional significance of increased miR-224 expression in the cells was evaluated by transfecting miR-224 precursor into HCT116 cells, which exhibit low endogenous miR-224 expression (data not shown). miR-

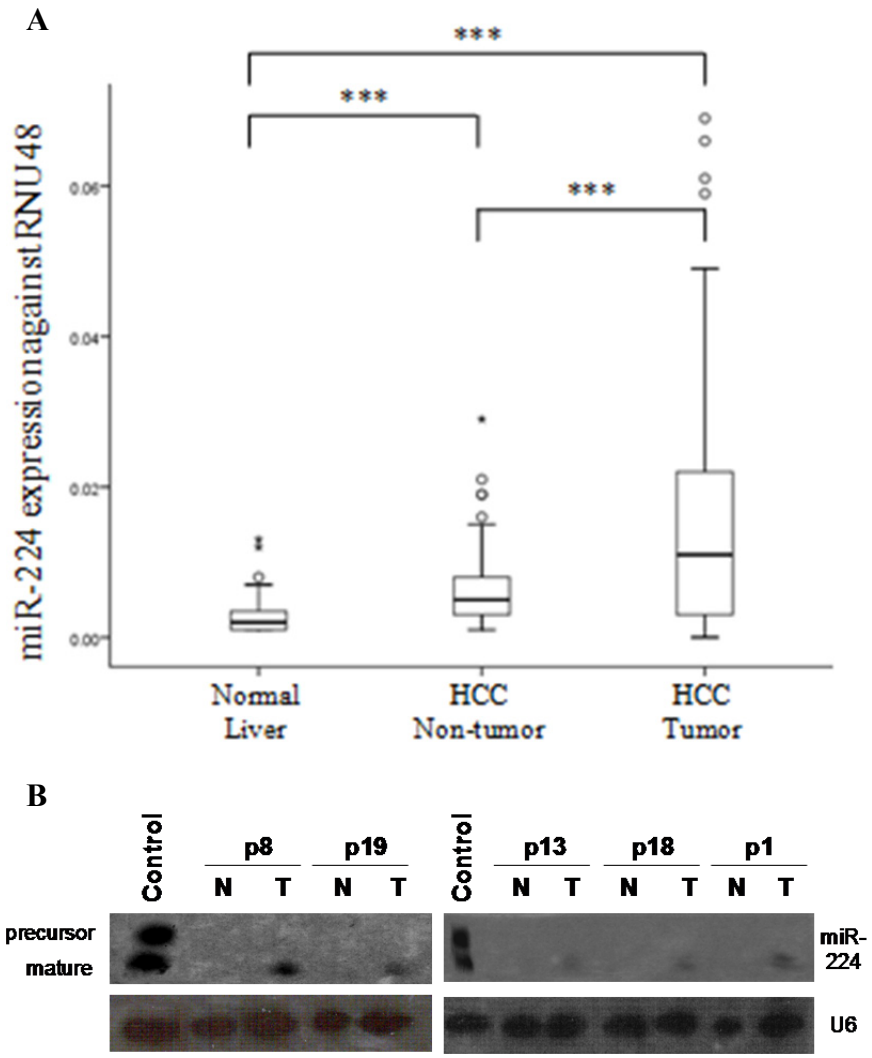

Figure 3.3.1 miR-224 is significantly up-regulated in tumors of HCC. (A) Box plot of miR-224 expression in the normal liver samples from 40 metastatic colorectal cancer patients (Normal Liver), the adjacent non-tumorous liver (HCC Non-tumor) and tumor tissues (HCC Tumor) from 100 HBV-associated HCC patients. *** denotes P<0.001. **(B)** Northern Blot analyses to validate the increased miR-224 expression in tumor tissues of representative HCC patients. 'P (number)' represents the identity of the patients examined through miRNA microarrays. 'T' denotes tumor tissue from indicated HCC patients while 'N' denotes the paired adjacent non-tumorous liver tissues. 'Control' represent total RNA isolated from HCT116 cells 72 hours after transfection with miR-224 precursor and hybridized with miR-224 probe.

(Published and unpublished data)

224 expression peaked 24 hours post transfection but its expression remained high (>100 fold higher than that of Control transfected cells) even at 168 hours post transfection (Figure 3.3.2.(1)). Some of the primary hallmarks of cancer including cell growth, viability and apoptotic profiles (37), were examined on miR-224 expressing cells. Cell growth remained generally unaffected in cells transfected with either miR-224 precursor or Control (Figure 3.3.2.(2)A) but cell viability was significantly reduced (up to 20% reduced cell viability; $P<0.05$) in cells transfected with miR-224 precursor compared to cells transfected with Control Oligo 2 (Figure 3.3.2.(2)B). These data suggest that miR-224 seems to affect the viability rather than the growth of these cells.

3.3.3 miR-224 over-expression sensitizes cells to apoptotic cell death

Annexin V labeling was performed to delineate if the reduced viability of cells over-expressing miR-224 is due to increased cell-death through apoptosis. miR-224 precursor- or Control-transfected HCT116 cells were harvested 48 hours post transfection, stained with PE-conjugated Annexin V and 7-AAD and analyzed on the flow cytometer. Apoptotic cells are represented as cells with high PE-conjugated Annexin V fluorescence signals and low 7-AAD fluorescence signals. As shown in Figure 3.3.3B, ~2 fold greater apoptosis was observed in cells expressing miR-224 than in cells expressing the control precursors ($P<0.001$). Significantly, when miR-224 inhibitor was introduced into cells over-expressing miR-224, the miR-224 expression was significantly reduced ($P<0.001$) (Figure 3.3.3A) and significantly less apoptosis ($P<0.001$) (Figure 3.3.3B) was observed, consistent with cells not over-expressing miR-224. Hence, the reduced cell-viability observed in miR-224-expressing cells is likely due to increased

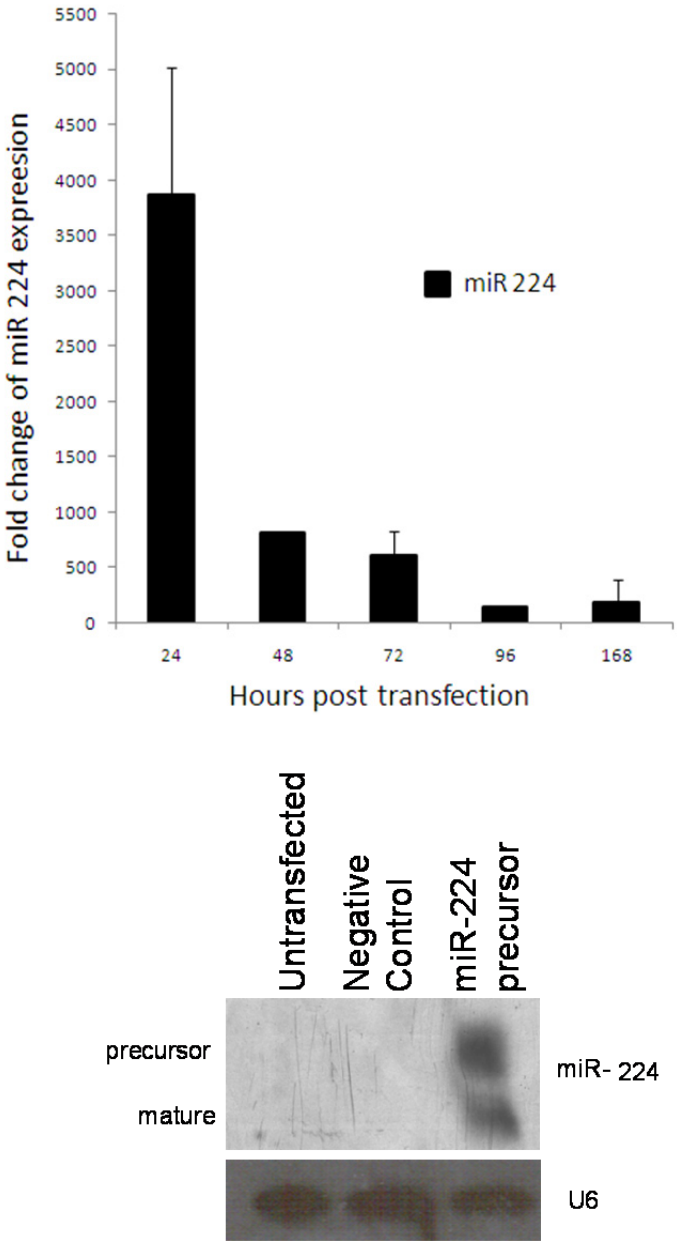

Figure 3.3.2.(1) miR-224 expression in HCT116 cells. *Upper panel* shows results from Taqman MicroRNA individual Assay (left) for hsa-miR-224 (Applied Biosystems) in HCT-116 cells after transfection with miR-224 and normalization against hsa-Let-7a for the various time-points. Results are expressed as fold difference between miR-224 expression in miR-224 precursor transfected cells versus Control transfected cells. *Lower panel* shows Northern Blot Analyses of untransfected HCT116 cells or HCT116 cells carrying control or miR-224 precursor, 72 hours after transfection.

(Published data)

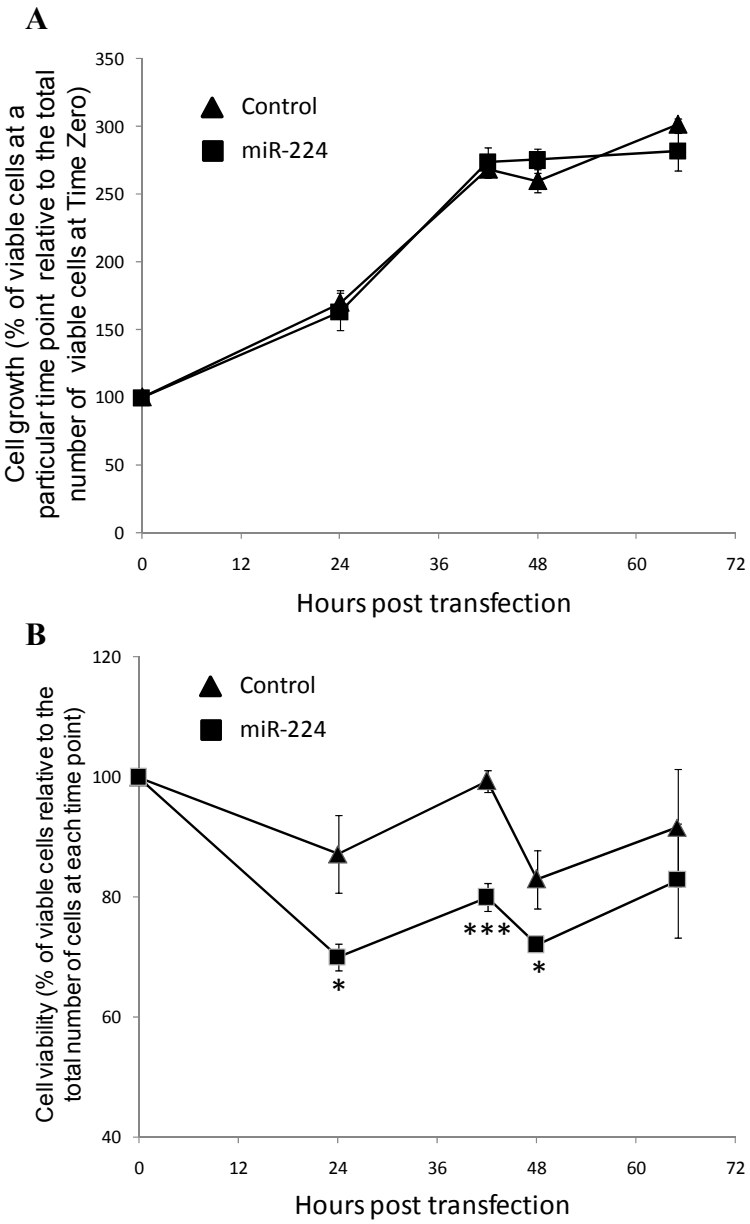

Figure 3.3.2.(2) Cell growth properties of miR-224 expressing cells. (A) Cell growth expressed as percentage of viable cells at the respective time points relative to the transfection start time, Time Zero. miR-224 precursor transfected cells are represented by squares and Control transfected cells are represented by triangles. **(B)** Viability of cells expressed as the percentage of viable cells relative to the total number of cells at a particular time point. Results were obtained by counting cells from the same experiment twice in three independent experiments. Data are as mean ± S.E. from at least 3 independent experiments. * denotes $p<0.05$, and *** denotes $P<0.001$.

(Published data)

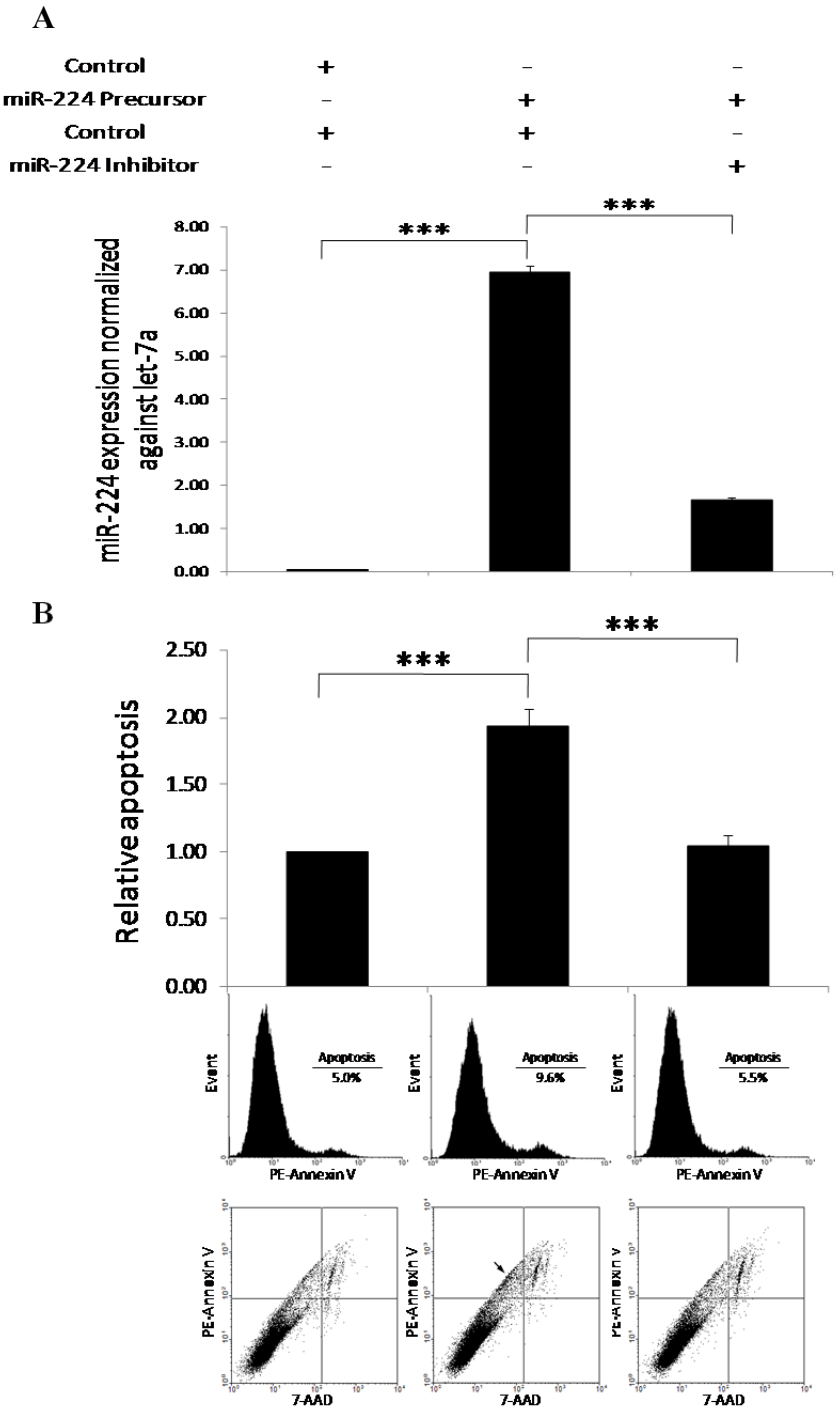

Figure 3.3.3 miR-224 increases apoptotic cell death. (A) miR-224 expression of cells transfected with Control, miR-224 precursors or miR-224 precursors and miR-224 inhibitors. **(B)** *Upper panel:* Relative Apoptosis of similarly treated cells, as assayed using PE-conjugated Annexin V staining. *Lower panel:* Apoptotic cells were detected as high in PE-annexin V staining and low in 7-AAD staining. Profiles shown are a representative from 3 independent experiments. Data are as mean ± S.E. from at least 3 independent experiments. *** denotes $P<0.001$.

(Published data)

apoptosis of these cells in the presence of miR-224 as evident from the increased apoptosis observed in miR-224 over-expressing cells and the restoration of its normal phenotype when miR-224 inhibitor was co-introduced.

3.3.4 miR-224 over-expression increases cell proliferation

Curiously, although miR-224 over-expression reduced cell-viability (Figure 3.3.2B) and increased apoptosis (Figure 3.3.3B), it did not seem to affect cell growth (Figure 3.3.2A), as measured by the percentage of viable cells at each time point relative to time zero. We thus hypothesized that this observation could be due to miR-224 also increasing cell proliferation in addition to increasing cell apoptosis, consequently resulting a similar number of viable cells in miR-224-expressing cells compared to control cells at any time point. The proliferation potential of these cells was then examined using the BrdU incorporation assay. As shown in Figure 3.3.4, transfection of miR-224 precursor into these cells significantly increased the proliferation potential of these cells ($P<0.001$) while the co-transfection of the miR-224 inhibitor with the miR-224 precursors restored the cell-proliferation potential to normal. Hence, the over-expression of miR-224 increases both apoptotic cell death as well as cell proliferation.

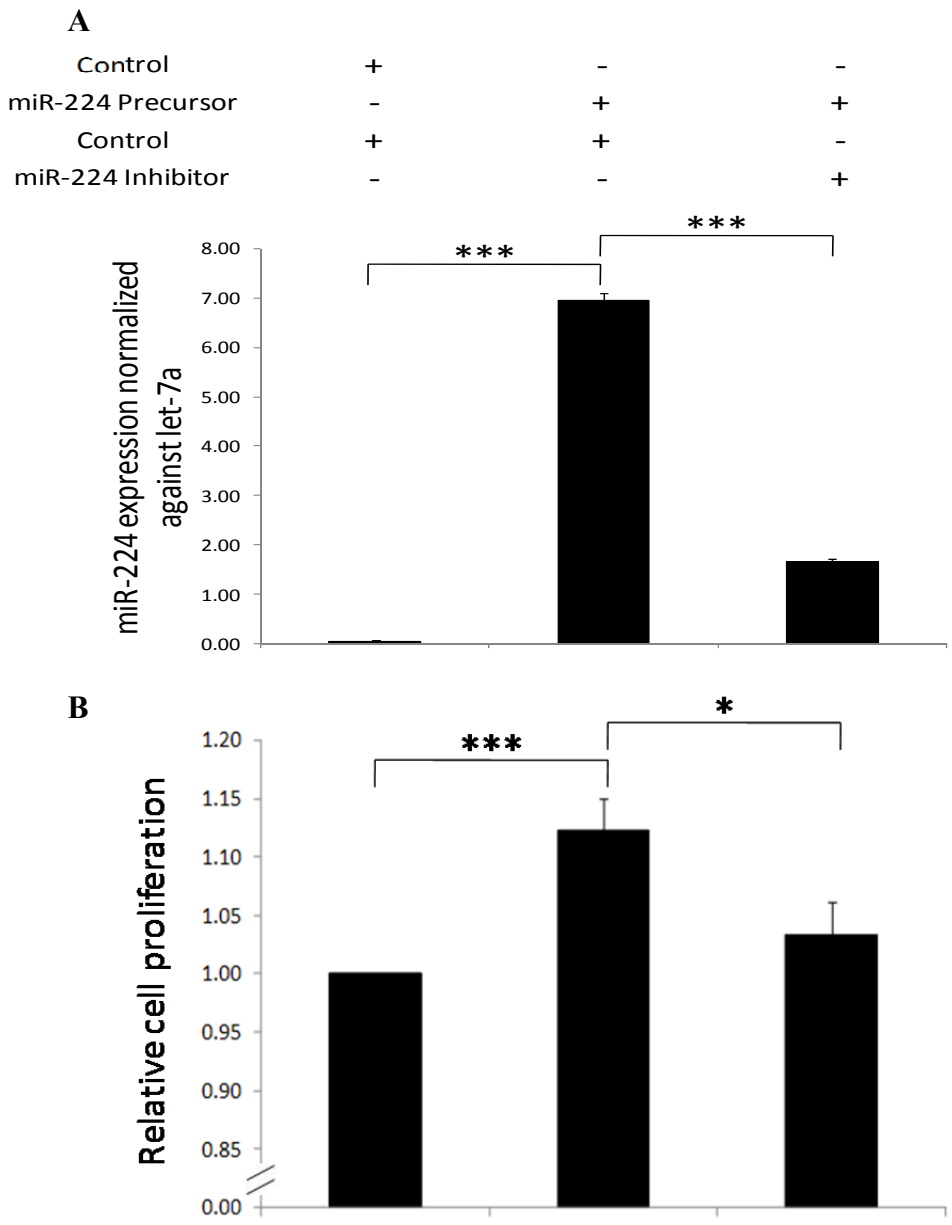

Figure 3.3.4 miR-224 increases cell proliferation. (A) miR-224 expression of cells transfected with Control, miR-224 precursors or miR-224 precursors and miR-224 inhibitors. **(B)** Relative Cell Proliferation of similarly treated cells assayed using the BrdU Proliferation Assay Kit. Data are as mean ± S.E. from at least 3 independent experiments. * denotes $p<0.05$ and *** denotes $P<0.001$.

(Published data)

3.3.5 miR-224 targets API-5

To elucidate the gene target that miR-224 acts through to sensitize cells to apoptosis, we examined the putative miR-224 targets as predicted by *in silico* miRNA target prediction algorithms. We used miRecords which is an online resource that compiles predicted miRNA targets produced by 11 established miRNA target prediction programs [256]. Six of these 11 prediction programs predict for human miR-224 targets and these are miRanda, mirTarget2, miTarget, PITA, RNAhybrid and TargetScan. Table 3.3.5 summarized the 52 putative miR-224 targets as predicted by at least 5 prediction programs and functionally annotated with Gene Ontology (GO) terms. Using Generic GO Term Mapper developed by the Lewis-Sigler Institute for Integrative Genomics, these 52 putative miR-224 targets were mapped into a total of 39 functional GO terms in biological processes with human generic GO slim as background. Many of these biological processes were associated with cancer, namely transcription, cell differentiation, cell death, growth and cell proliferation. As miR-224 increases apoptotic cell death, we focused on putative miR-224 targets that are negative regulators of cell death. Five gene targets were identified to be involved in cell death as boxed in red in Table 3.3.5. Of these five putative miR-224 gene targets, apoptosis inhibitor 5 (API-5) seems to be the most appropriate candidate through which miR-224 may act to increase apoptosis in cells since API-5 has previously been reported to be an anti-apoptotic gene (40-42).

(Unpublished data)

Table 3.3.5 GO term enrichment of putative miR-224 targets predicted by at least 5 prediction algorithms

GO Term	GO ID	Genes Annotated to the GO term
Metabolic process	0008152	ACSL4, AFF3, ATF2, BRPF3, BTRC, CASC3, DPYSL2, HMGCR, MAPK14, MED13L, NCOA6, NUAK1, POLR1D, POU3F2, PPAP2B, PRPF4B, RAD51L1, RUNX2, SDC4, SMAD4, SR140, TCERG1, TRIM9, UBE2D3, WTAP, YOD1, ZNF207, ZNF423
Primary metabolic process	0044238	ACSL4, AFF3, ATF2, BRPF3, BTRC, CASC3, DPYSL2, HMGCR, MAPK14, MED13L, NCOA6, NUAK1, POLR1D, POU3F2, PPAP2B, PRPF4B, RAD51L1, RUNX2, SMAD4, SR140, TCERG1, TRIM9, UBE2D3, WTAP, YOD1, ZNF207, ZNF423
Regulation of biological process	0050789	AFF3, AMIGO2, API5, ATF2, BTRC, CASC3, DPYSL2, ITM2B, MAP1B, MAPK14, MED13L, NCOA6, POU3F2, PPAP2B, RAB10, RUNX2, SDC4, SMAD4, TCERG1, UBE2D3, ZNF207, ZNF423
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0006139	AFF3, ATF2, CASC3, DPYSL2, HMGCR, MED13L, NCOA6, POLR1D, POU3F2, PPAP2B, PRPF4B, RAD51L1, RUNX2, SMAD4, SR140, TCERG1, UBE2D3, WTAP, ZNF207, ZNF423
Multicellular organismal development	0007275	ACSL4, AFF3, BTRC, DPYSL2, GGNBP2, H3F3B, HMGCR, ITM2B, MAP1B, NCOA6, POU3F2, PPAP2B, RUNX2, SDC4, SMAD4, ZNF423
Biosynthetic process	0009058	AFF3, ATF2, CASC3, HMGCR, MED13L, NCOA6, POLR1D, POU3F2, PPAP2B, RUNX2, SMAD4, TCERG1, ZNF207, ZNF423
Transcription	0006350	AFF3, ATF2, MED13L, NCOA6, POLR1D, POU3F2, PPAP2B, RUNX2, SMAD4, TCERG1, ZNF207, ZNF423
Protein metabolic process	0019538	BRPF3, BTRC, CASC3, MAPK14, NUAK1, PPAP2B, PRPF4B, SMAD4, TRIM9, UBE2D3, YOD1
Cellular component organization	0016043	ACSL4, BRPF3, DIAPH3, H3F3B, MAP1B, NCOA6, POU3F2, SDC4, SMAD4
Cell differentiation	0030154	ACSL4, DPYSL2, GGNBP2, MAP1B, NCOA6, POU3F2, RUNX2, SMAD4, ZNF423
Protein modification process	0006464	BRPF3, BTRC, MAPK14, NUAK1, PPAP2B, PRPF4B, SMAD4, UBE2D3, YOD1
Response to stress	0006950	CASC3, MAP1B, MAPK14, NCOA6, RAD51L1, SMAD4, UBE2D3, YOD1
Catabolic process	0009056	BTRC, CASC3, SMAD4, TRIM9, UBE2D3, YOD1
Death	0016265	AMIGO2, **API5**, ITM2B, MAPK14, UBE2D3
Anatomical structure morphogenesis	0009653	BTRC, MAP1B, POU3F2, PPAP2B, SMAD4
Signal transduction	0007165	BTRC, DPYSL2, MAPK14, RAB10, SMAD4
Cell communication	0007154	BTRC, DPYSL2, MAP1B, POU3F2, SMAD4
Embryo development	0009790	ACSL4, MAP1B, NCOA6, PPAP2B, SMAD4
Cell death	0008219	AMIGO2, **API5**, ITM2B, MAPK14, UBE2D3
Organelle organization	0006996	BRPF3, DIAPH3, H3F3B, MAP1B, SDC4
Reproduction	0000003	ACSL4, GGNBP2, HMGCR, PPAP2B
Response to endogenous stimulus	0009719	DPYSL2, H3F3B, MAP1B, NCOA6
DNA metabolic process	0006259	NCOA6, RAD51L1, UBE2D3
Protein transport	0015031	GGA3, RAB10, SMAD4
Response to external stimulus	0009605	ACSL4, MAP1B, MAPK14
Cytoskeleton organization	0007010	DIAPH3, MAP1B, SDC4
Lipid metabolic process	0006629	ACSL4, HMGCR, PPAP2B
Cell proliferation	0008283	BTRC, POU3F2, **SMAD4**
Growth	0040007	MAP1B, POU3F2, **SMAD4**
Cell cycle	0007049	BTRC, RAD51L1, WTAP
Behavior	0007610	ACSL4, MAPK14
Cell growth	0016049	MAP1B, **SMAD4**
Cell-cell signaling	0007267	DPYSL2, MAP1B
Ion transport	0006811	KCTD12, SLC4A4
Response to abiotic stimulus	0009628	MAP1B
Cellular amino acid and derivative metabolic process	0006519	YOD1
Response to biotic stimulus	0009607	YOD1
Translation	0006412	CASC3
Cellular homeostasis	0019725	POU3F2

3.3.5.(1) miR-224 specifically interacts with the putative miR-224 binding site along API5 3'UTR to negatively regulate reporter gene expression.

We proceeded to validate whether API-5 was in fact a true gene target of miR-224. As illustrated in Figure 3.3.5.(1)A, three miR-224 binding sites were identified along the 2035-bp long 3'UTR of API-5. The entire wild-type 3'UTR of API-5 as well as a mutant 3'UTR where all three putative miR-224 binding sites were mutated was then cloned downstream the β-galactosidase (β-gal) reporter gene to generate pAPI5-3UTR-WT and pAPI5-3UTR-MUT, respectively. The specific interaction between miR-224 and API-5 3'UTR to inhibit reporter gene activity was evident in Figure 3.3.5.(1)B. When miR-224 precursor was co-transfected with wild-type API-5 3'UTR reporter construct, significantly reduced β-gal activity was observed compared to cells co-transfected with miR-224 precursor and mutant API-5 3'UTR reporter construct ($P<0.01$). No significant difference in β-gal activity was observed between HCT116 cells carrying either the wild-type or mutant API-5 3'UTR reporter construct when co-transfected with Control Oligos.

3.3.5.(2) miR-224 negatively regulates endogenous API5 expression.

To evaluate if miR-224 can affect the endogenous expression of API-5, HCT116 cells were initially transfected with either miR-224 precursor or Control. miR-224 inhibitor or Control were then introduced into these transfected cells 24 hours later and reverse transcription real-time PCR was performed at various time points after this 2nd transfection. As shown in Figure 3.3.5.(2), API-5 expression was significantly lower in miR-224

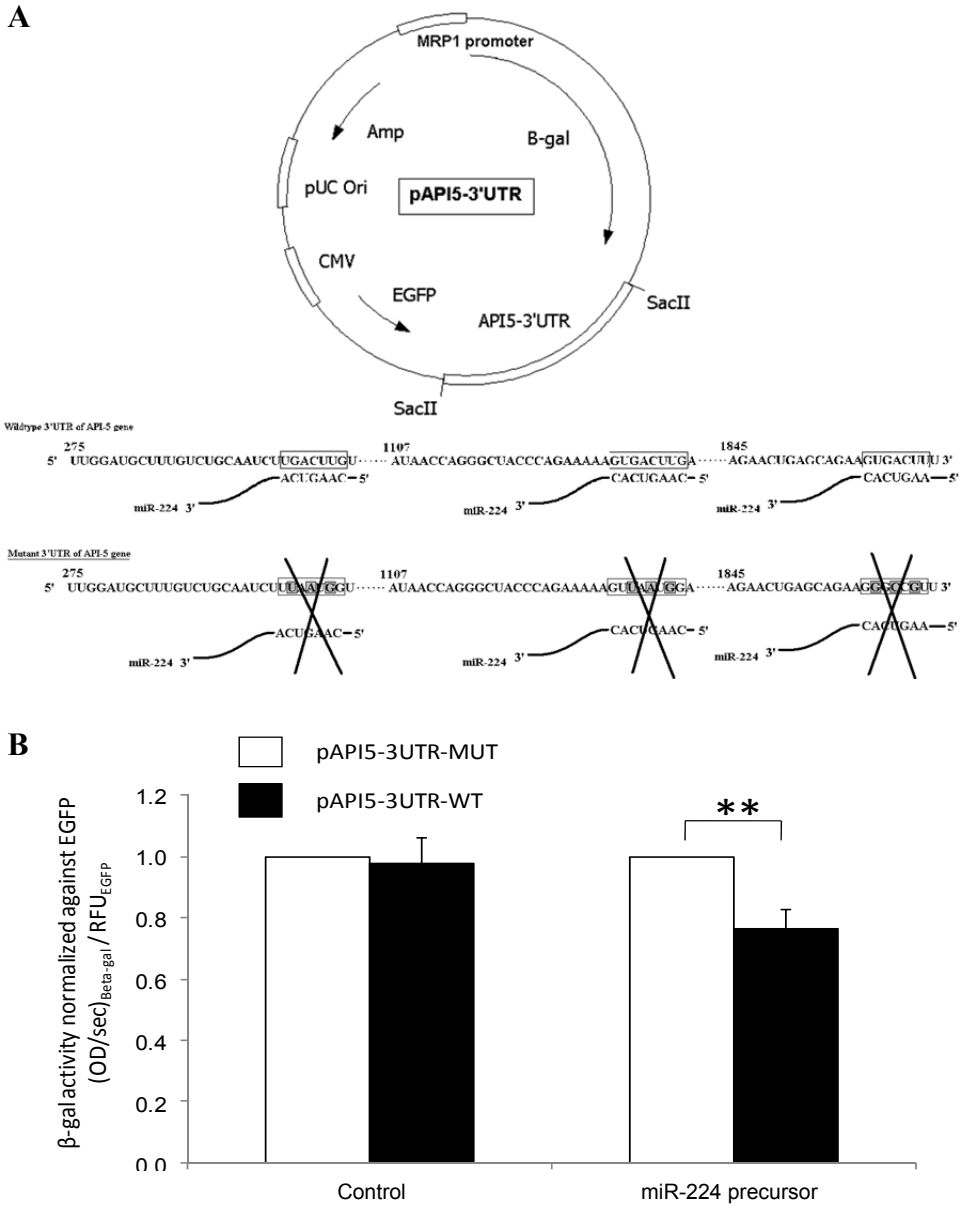

Figure 3.3.5.(1) miR-224 interacts with the putative binding sites on API5 3' UTR. (A) *Upper panel:* Schematic diagram of the construct utilized to validate that the API-5 3'UTR is target of miR-224. The API-5 3'UTR (wild-type or mutant) is cloned downstream the β-gal reporter driven by MRP1 promoter. *Lower panel:* Schematic representation of the 3' UTR of API-5 in which the predicted sequences of the three miR-224 binding sites are mutated to abolish miR-224 binding to the API-5 3'UTR. **(B)** Action of miR-224 on the 3'UTR of API-5 examined through normalized β-gal reporter activities in cells cotransfected with miR-224 precursors or Control and wild-type API-5 3'UTR (black bar) or mutant API-5 3'UTR (white bar) reporter constructs. Data presented as Mean±SE from at least three independent experiments. ** denotes $p<0.01$.

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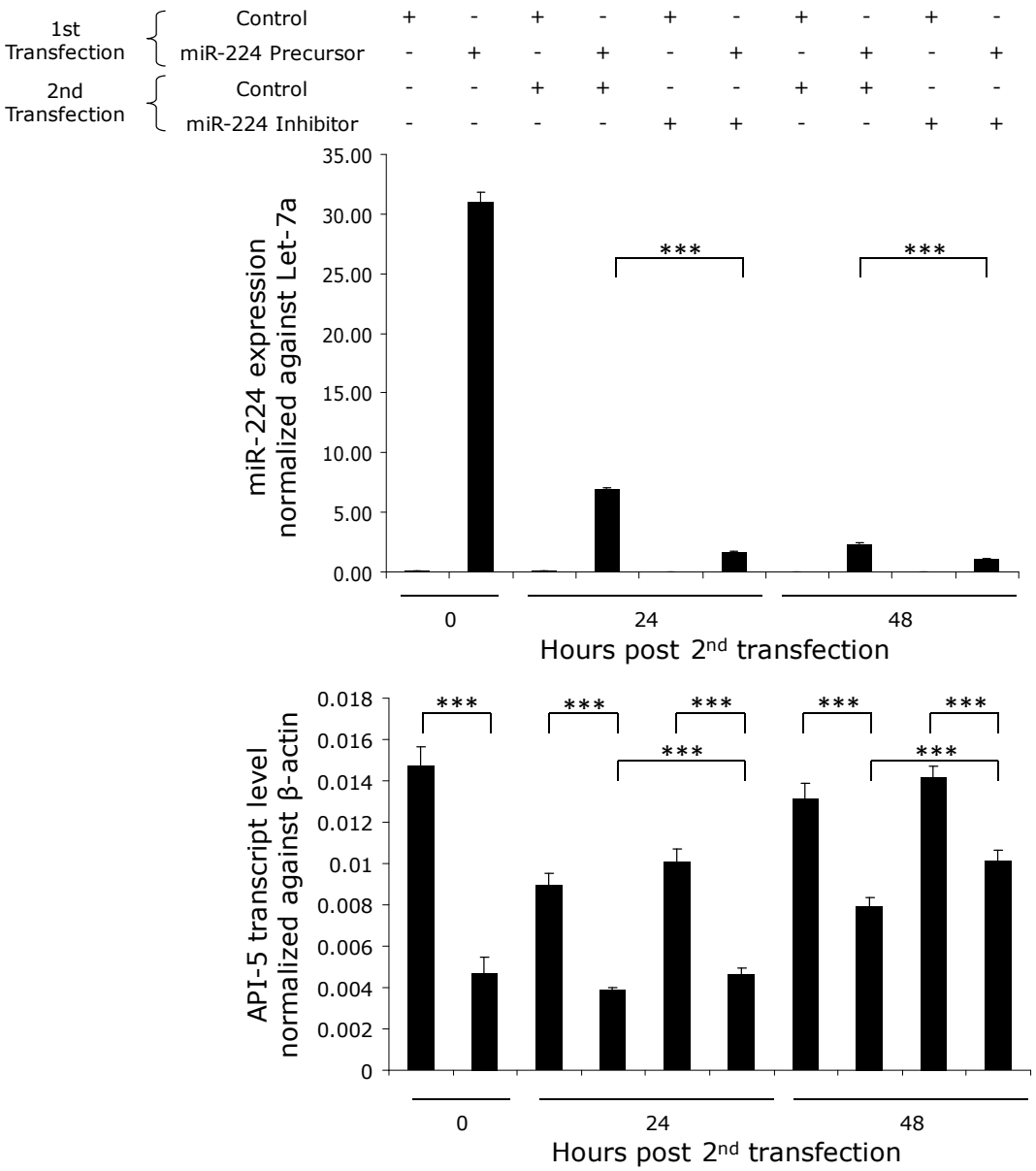

Figure 3.3.5.(2) miR-224 negatively regulates API-5 expression. miR-224 expression (normalized against RNU48) (Top) and endogenous API-5 mRNA expression (normalized against β-actin) (bottom) in control or miR-224 precursor and miR-224 inhibitor transfected cells. Cells were initially transfected with either Control or miR-224 precursors. Twenty-four hours later, the same cells were transfected with either Control or miR-224 inhibitors. miR-224 and API-5 RNA levels were measured at various time-points after the 2nd transfection. Data are represented as Mean ± S.E. from at least three independent experiments. *** denotes $P<0.001$.

(Published data)

precursor transfected HCT116 cells compared to the control cells ($P<0.001$) across time points examined, consistent with previous reports that miRNAs can also down-regulate gene expression at the transcript level (45). When miR-224 inhibitor was transfected into miR-224 over-expressing cells, the API-5 transcript levels significantly increased ($P<0.001$) at the 2 time points examined suggesting that the miR-224 inhibitor was able to rescue the inhibition of API-5 expression by miR-224. This observation that miR-224 decreased API-5 expression corroborates with our earlier observation that miR-224 increases apoptotic cell death and strongly suggests that miR-224 exerts this effect through regulating the expression of API-5.

3.3.6 Inhibition of miR-224 in THLE3 cells up-regulates API-5 expression and reduces apoptotic cell death.

Thus far, we observed that miR-224 is over-expressed in the majority of HCC patients examined and demonstrated experimentally that the over-expression of miR-224 in HCT116 cells increased apoptosis and reduced expression of the apoptosis inhibitor, API-5 gene. We proceeded to evaluate the functional consequences of inhibiting the endogenous expression of miR-224 in an immortalized primary liver cell-line, THLE-3. The introduction of miR-224 inhibitor into THLE-3 cells was found to significantly inhibit the endogenous expression of miR-224 in these cells ($P<0.001$) (Figure 3.3.6(A), left) and significantly increased the expression of the API-5 gene (Figure 3.3.6A, right). Inhibiting endogenous miR-224 expression in the THLE-3 primary liver cells was also found to protect these cells from UV-induced apoptotic cell death ($P<0.05$) (Figure 3.3.6B).

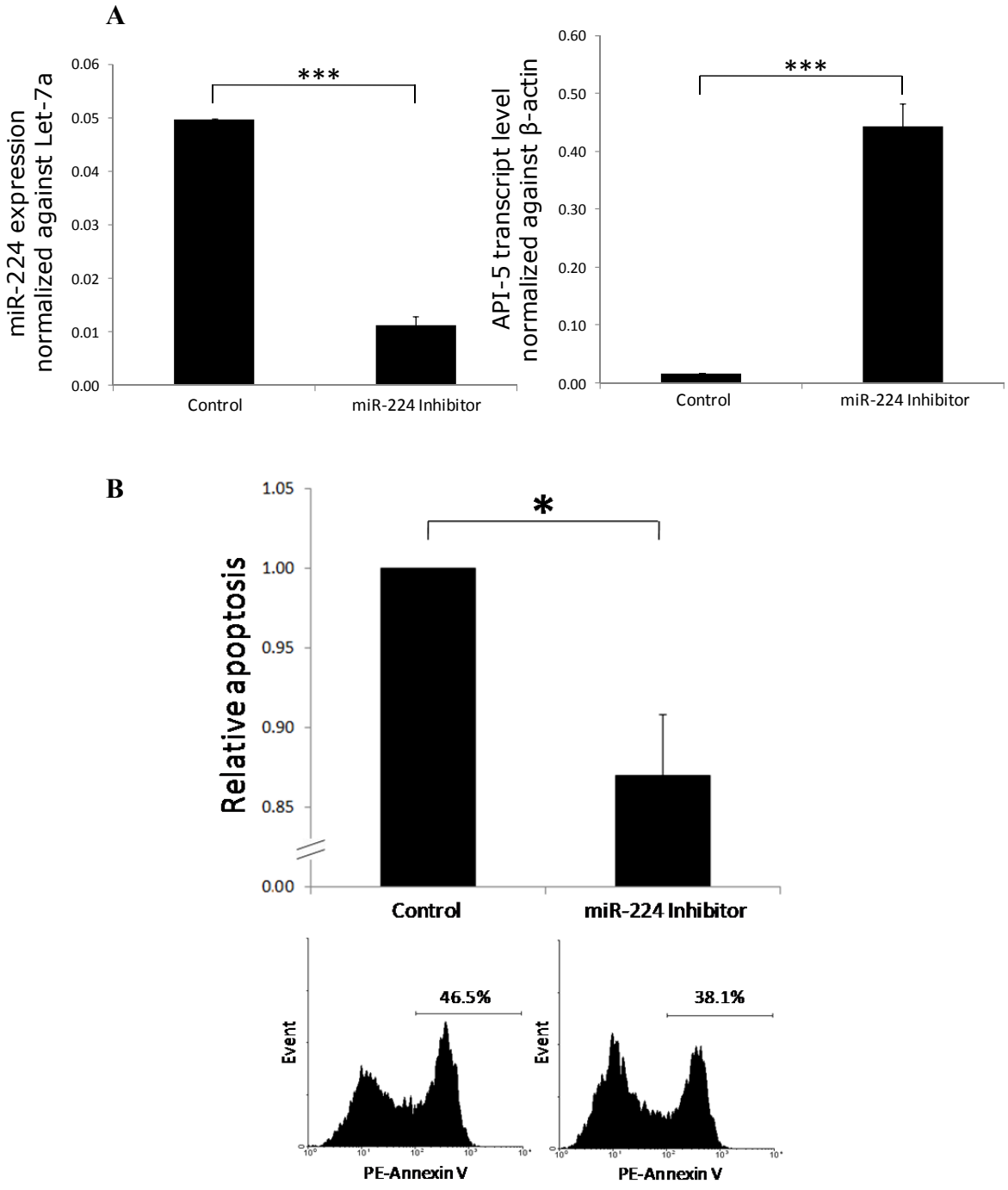

Figure 3.3.6 Knock-down of miR-224 in THLE3 cells decreased the number of apoptotic cells. (A) miR-224 (Left) and *API-5* (Right) expression in THLE-3 cells transfected with either Control or miR-224 inhibitor. **(B)** Relative apoptosis of THLE-3 cells transfected with either Control or miR-224 inhibitor. Results show data from at least 3 independent experiments, expressed as mean ± S.E., * denotes $P<0.05$ and *** denotes $P<0.001$.

(Published data)

3.3.7 API-5 mRNA expression was inversely correlated with miR-224 expression in HCC patients

We proceeded to examine if there is any correlation between miR-224 and API-5 expression in HCC patients to evaluate the clinical significance of our observations. As evident in Figure 3.3.7, a statistically significant inverse correlation was observed between miR-224 and API-5 expression in HCC patients (Pearson Coefficient $r=-0.471$, $R^2=0.221$ at $p< 0.05$), demonstrating API5 as a clinically relevant target of miR-224, in HCC context.

3.3.8 miR-224 targets SMAD4 to affect cell proliferation

To identify the cellular target through which miR-224 acts to increase cell proliferation, we employed the similar hypothesis driven approach. As shown by Table 3.3.5, a total of four non-redundant genes were identified to be involved in either cell proliferation (GO008283), growth (GO004007) or cell growth (GO0016049) (Boxed in blue). SMAD4 was the only putative target appeared in all three categories and a highly probable target through which miR-224 positively regulates cell proliferation. SMAD4 is the central mediator for Transforming Growth Factor beta (TGF-β) pathway which negatively regulates cell proliferation. Hypothetically, the possible down-regulation of SMAD4 by miR-224 over-expression will de-repress the TGF-β mediated check on cell growth, resulting increased cell proliferation.

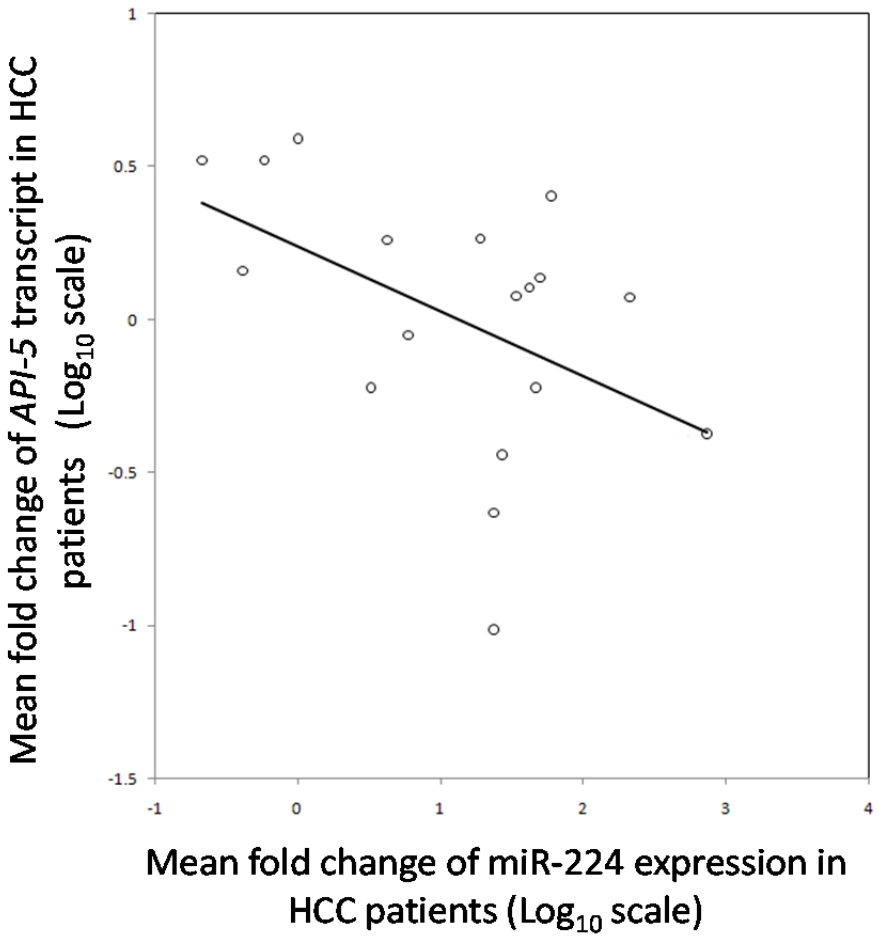

Figure 3.3.7 API-5 transcript expression level is inversely correlated with miR-224 expression in HCC patients. Scatter plot of the mean fold change of API-5 transcript versus that of miR-224 expression. Each spot represents data from one patient presented in $\log_{10}$ scale and a linear regression line is depicted as a solid line. Statistically significant inverse correlation was observed between API-5 and miR-224 expression ($p<0.05$ and $R^2=0.221$).

(Published data)

3.3.8.(1) miR-224 specifically interacts with the putative miR-224 binding site along SMAD4 3'UTR to negatively regulate reporter gene expression.

To elucidate whether miR-224 directly targets SMAD4, we first identified two putative miR-224 binding sites along SMAD4 3'UTR with miRanda prediction program (www.microRNA.org). We subsequently cloned the 1310 bp long wildtype SMAD4 3'UTR as well as a mutant SMAD4 3'UTR in which the putative miR-224 binding sites were mutated to abolish miR-224 binding, downstream of the β-galactosidase reporter gene driven by MRP1 promoter. As evident in Figure 3.3.8.(1), when miR-224 precursors were introduced, cells carrying the wildtype SMAD4 3'UTR reporter construct showed significantly reduced β-galactosidase activity ($p<0.01$) compared to the cells carrying the mutant SMAD4 3'UTR reporter construct in which the miR-224 bindings were abolished. However, no significant difference in reporter gene activity was observed between cells carrying the wildtype or mutant SMAD4 3'UTR reporter construct, when Control Oligos were introduced. This data suggested that miR-224 specifically interacted with the putative binding sites along the SMAD4 3'UTR to negatively regulate reporter gene activity.

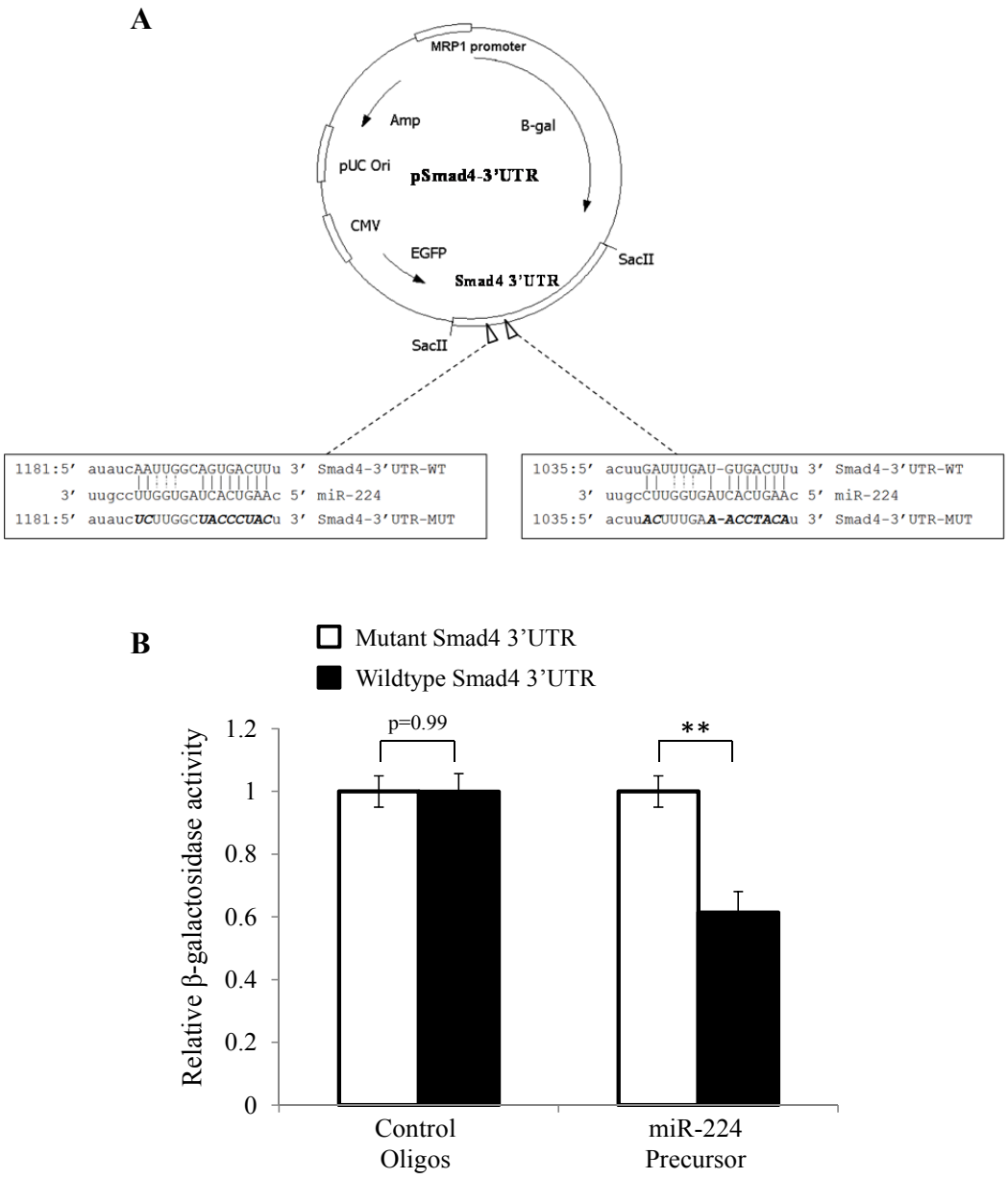

Figure 3.3.8.(1) miR-224 interacts with putative binding sites on SMAD4 3'UTR. (A) Schematic diagram of the constructs utilized to validate that the miR-224 targets the 3'UTR region of SMAD4. The wildtype SMAD4 3'UTR carrying the predicted miR-224 binding sites (Upper panel in the box) or the mutant SMAD4 3'UTR carrying the mutated miR-224 binding sites (Lower panels with the mutation residues in BOLD) were cloned downstream β-galactosidase reporter gene driven by MRP1 promoter. **(B)** Effect of miR-224 on SMAD4 3'UTR examined through normalized β-galactosidase activity in cells co-transfected with Control oligos or miR-224 precursor and wildtype SMAD4 3'UTR (Black bar) or mutant SMAD4 3'UTR reporter construct (White bar). Data presented as Mean±S.E from at least three independent experiments. ** denotes $p<0.01$.

(Unpublished data)

3.3.8.(2) miR-224 negatively regulates endogeneous SMAD4 expression.

To evaluate if miR-224 can affect the endogenous expression of SMAD4, we manipulated the endogeneous miR-224 expression using specific miR-224 precursors and inhibitors. As shown in Figure 3.3.8.(2), SMAD4 expression was significantly lower ($p<0.001$, lower panel) in HCT116 cells transfected with miR-224 precursor to over-express miR-224 ($p<0.001$, top panel), compared to the Control Oligos transfected cells. When miR-224 inhibitor was co-transfected with miR-224 precursors, the over-expression of miR-224 was partially rescued ($p<0.001$, top panel) and the SMAD4 transcript level was significant increased ($p<0.01$) compared to that of the miR-224 precursor transfected cells, suggesting that the miR-224 inhibitor was able to rescue the inhibition of SMAD4 expression by miR-224.

3.3.8.(3) SMAD4 inhibition phenocopies miR-224 over-expression.

To ascertain that SMAD4 is indeed a target through which miR-224 affects cell proliferation, we inhibited SMAD4 expression with siRNA which specifically targets SMAD4 (si-SMAD4) and examined its effect on cell proliferation. As shown in Figure 3.3.8.(3)B, SMAD4 inhibition increased cell proliferation (rightmost lane), phenocopying the effect of overexpression of miR-224 (middle lane). Figure 3.3.8.(3)A shows the expression of miR-224 and SMAD4 when miR-224 precursor and si-SMAD4 were introduced into the cells. Taken together, our data demonstrated that miR-224 regulates cell proliferation at least partially through its direct target, SMAD4.

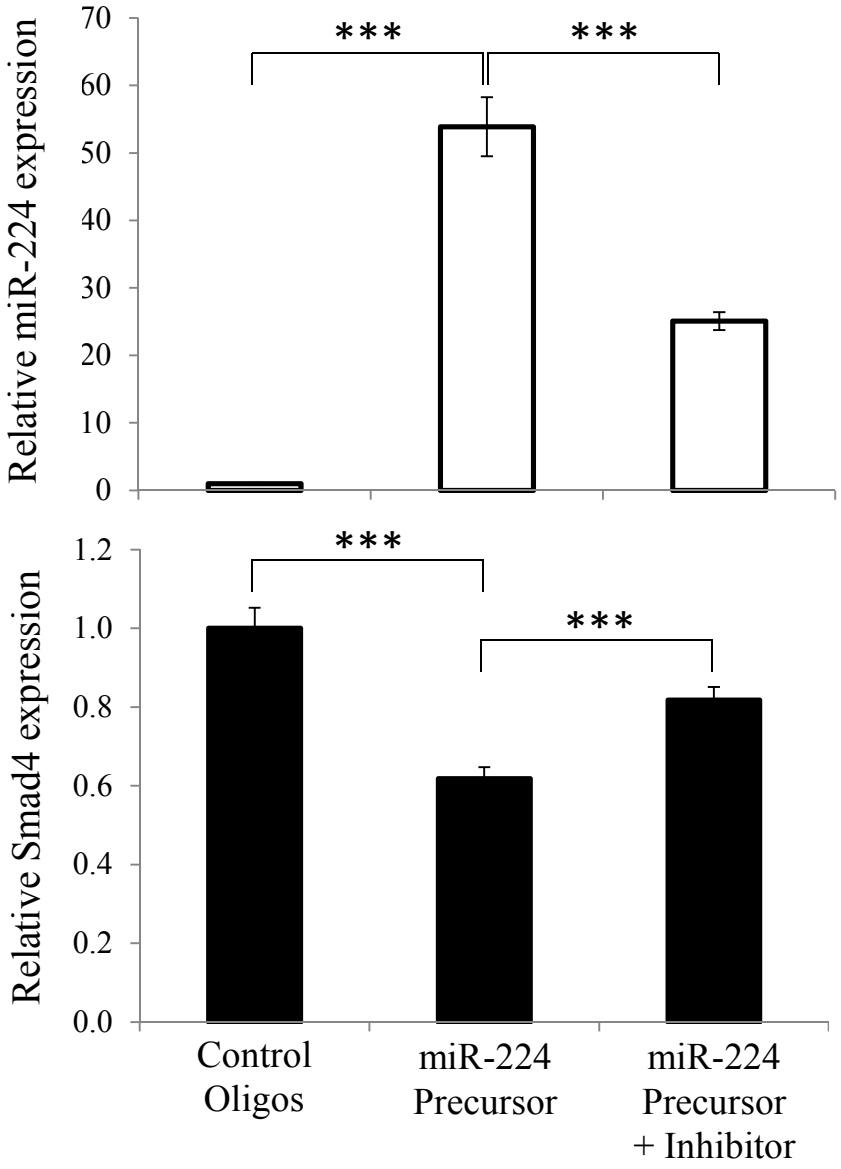

Figure 3.3.8.(2) miR-224 negatively regulates SMAD4 transcript expression. Relative miR-224 expression (normalized against RNU48) (Top) and relative SMAD4 transcript expression (normalized against β-actin) (bottom) measured with RT-qPCR in HCT116 cells transfected with Control Oligos or miR-224 precursor or co-transfected with both miR-224 precursor and inhibitor. Data are represented as Mean ± S.E. from at least three independent experiments. *** denotes $P<0.001$.

(Unpublished data)

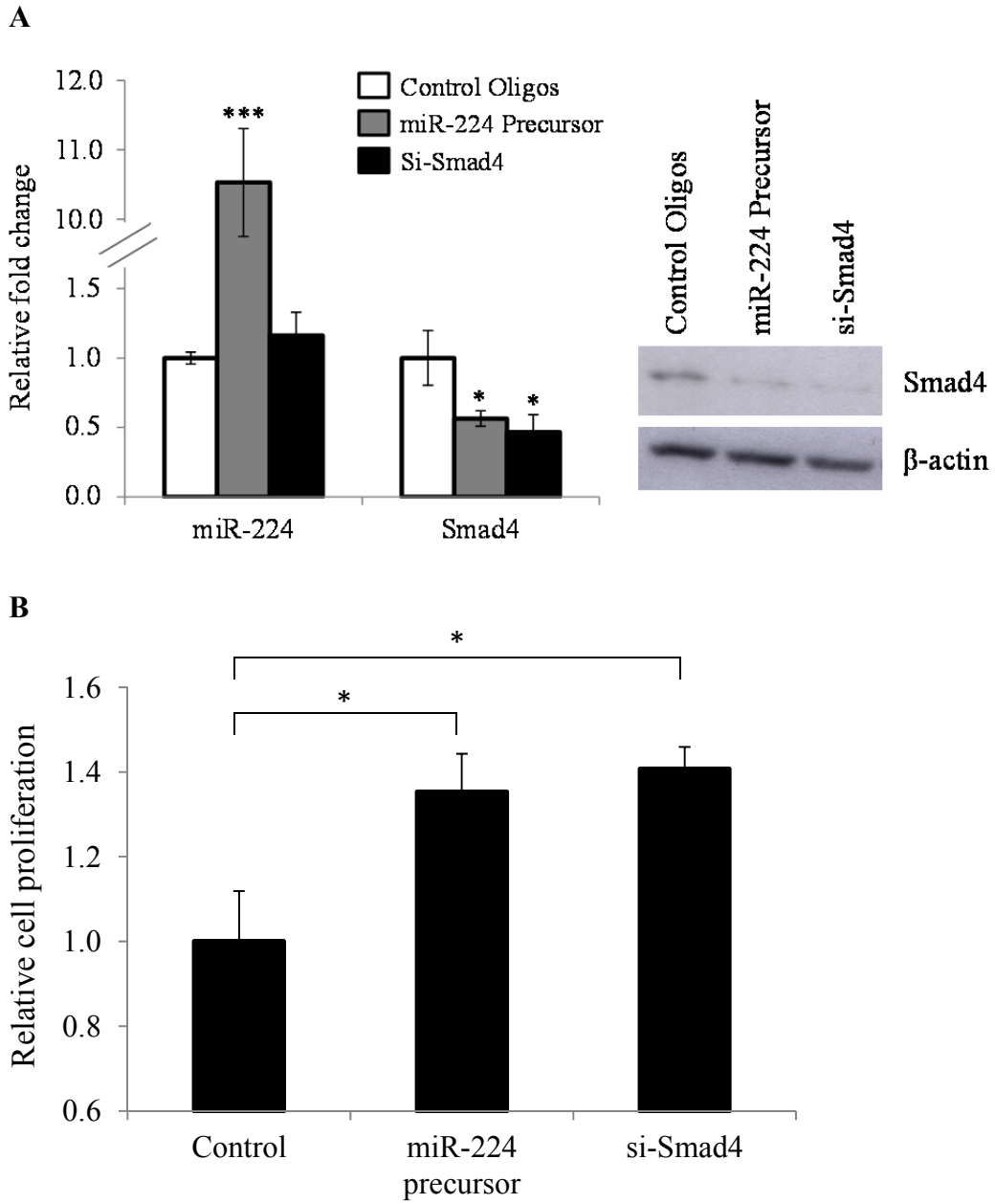

Figure 3.3.8.(3) Cells whose SMAD4 expression is inhibited shows similar proliferation phenotype as cells over-expressing miR-224. (A) Expression of miR-224 and SMAD4 after the introduction of either miR-224 precursors or siRNA against SMAD4, measured with RT-qPCR (left panel) and Western blot analysis (right panel). **(B)** Relative cell proliferation of HepG2 cells transfected with Control oligos, miR-224 precursor or siRNA against SMAD4 measured using the BrdU proliferation assay. Data presented as Mean ± SE from at least three independent experiments. * denotes $p<0.05$ and *** denotes $p<0.001$.

(Unpublished data)

3.3.9 SMAD4 mRNA expression was inversely correlated with miR-224 expression in HCC patients

We proceeded to evaluate the clinical significance of our observations that miR-224 negatively regulates SMAD4 by carrying out a correlation analysis between miR-224 and SMAD4 transcript expression in 100 HCC patients. As evident in Figure 3.3.9, a statistically significant inverse correlation was observed between miR-224 and SMAD4 expression in HCC patients (Pearson Coefficient $r=-0.350$, $R^2=0.122$ at $p< 0.001$), demonstrating SMAD4 as another clinically relevant direct cellular target of miR-224 in HCC context.

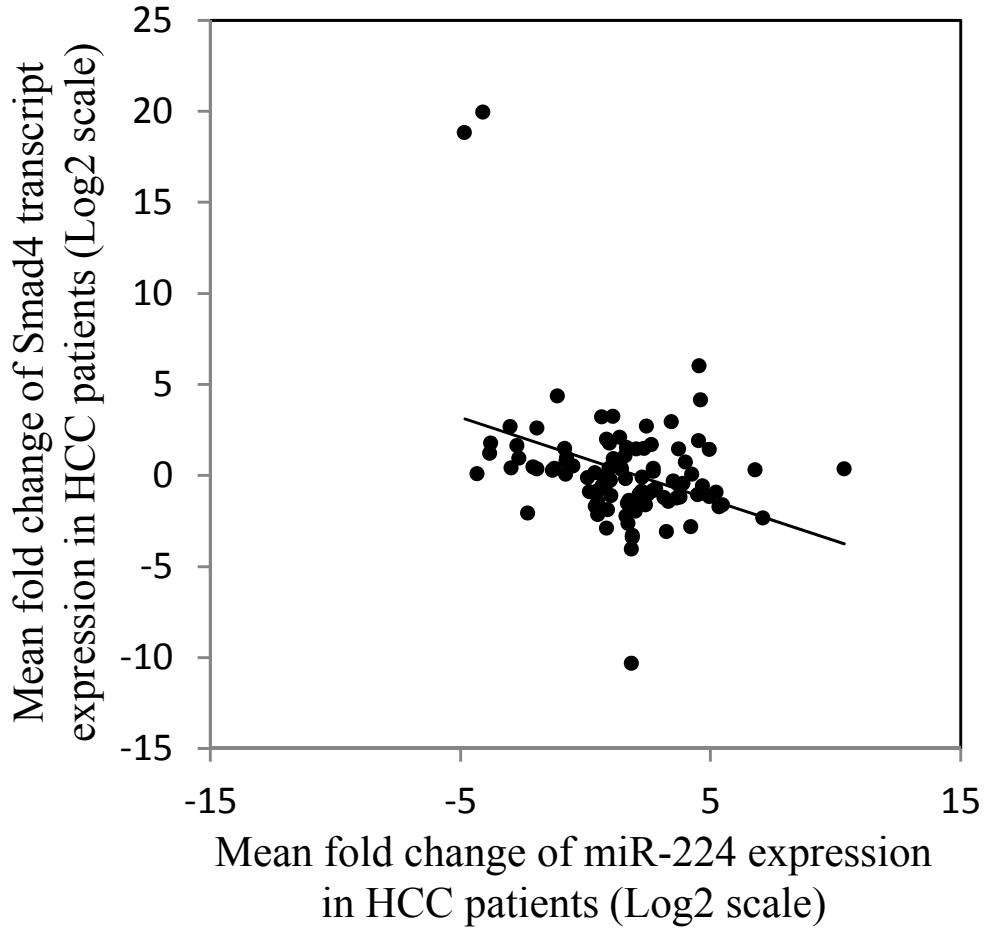

Figure 3.3.9 SMAD4 transcript expression level is inversely correlated with miR-224 expression in HCC patients. Scatter plot of the mean fold change of SMAD4 transcript versus that of miR-224 expression. Each spot represents data from one patient presented in $\log_2$ scale and a linear regression line is depicted as a solid line. Statistically significant inverse correlation was observed between SMAD4 and miR-224 expression ($p<0.001$, $R= -0.35$ and $R^2=0.122$).

(Unpublished data)

CHAPTER 4: DISCUSSION

4.1 miRNA deregulation in hepatocellular carcinoma

Like other cancers, aberrant gene expression features significantly in human hepatocellular carcinoma [260]. At the time when this project started, miRNAs, the largest class of gene regulators at the post-transcriptional and translational level, were found to be significantly deregulated in a number of human blood cancers [44] and solid tumors [74]. However, comprehensive profiling of miRNA expression in human HCC was still very limited. Therefore, we aim to identify significant miRNA deregulation by profiling the miRNA expression in local HCC patients. As described in Section 3.1 in the Results, we profiled tumor versus paired adjacent non-tumorous tissues from 100 HCC patients and identified 66 significantly differentially expressed miRNAs associated with HCC. As shown by Table 3.1, 36 of these 66 miRNAs have been previously reported to be differentially expressed in HCC. We observed 97 % (35 out of 36 miRNAs) consistency between our data and the previously reported data, demonstrating the reliability and accuracy of our miRNA profiling revealing true HCC information that is not confounded by the patients selected. In addition, we have also identified 30 novel miRNA deregulations in HCC with majority of these miRNAs absent from the miRNA profiling in previously reported studies. Hence, our work reaffirms current knowledge on miRNA deregulation in HCC and added new targets and insights with the identification of novel miRNA deregulation that was not identified previously.

These 66 significantly differentially expressed miRNAs in HCC were not randomly distributed in the genome but rather clustered based on the genomic loci or their functional miRNA family. These miRNA clusters show evidence of coordinated expression in our HCC samples, suggesting these deregulated miRNAs may be coordinately regulated based on either genomic loci or family-defining seed sequences.

In Section 3.2.1 to 3.3.3, we have provided some experimental evidence to partly support the above hypothesis. We observed significant down-regulation of members of let-7 family of miRNAs (let-7a, let-7b and let-7c) in HCC tumors and this let-7 down-regulation was significantly inversely correlated with expression status of HBV viral X protein (HBx) in HCC patients. To ascertain whether HBx plays a more direct role in down-regulating let-7, we transiently introduced HBx in HepG2 cells and observed direct down-regulation of let-7 miRNAs. As the focus of this project is to understand the functional relevance of the observed miRNA deregulation in HCC, we did not further characterize the exact mechanism of how HBx down-regulates let-7. As these let-7 miRNAs reside on different genomic loci, we speculate that HBx may down-regulate let-7 by targeting the let-7 specific family-defining seed sequence at the stage of miRNA processing. LIN28B is identified and characterized in HCC [261] and functions to block let-7 precursors from being processed into mature miRNAs by inducing terminal uridylation and degradation of let-7 precursors [262]. LIN28B is frequently over-expressed in HCC and may explain for the reduced let-7 expression in HCC tumors. High LIN28B expression and low let-7 expression are associated with poor clinical prognosis [263]. It would be interesting to explore if HBx

acts through LIN28 to negatively regulate let-7 maturation. The identification of cellular factors which are responsible for regulating families of miRNAs may represent novel targets through which novel strategies can be devised to the reverse the coordinated deregulation of such miRNAs.

In addition of the deregulation of miRNA families, we also found that 32 out of these 66 deregulated miRNAs reside in miRNA clusters in the human genome. 16 of these 32 clustered miRNAs (50 %) showed evidence of coordinated expression at 7 genomic clusters (Figure 3.1 and Table 3.1), suggesting common mechanisms of co-regulation at these genomic loci. Possible mechanisms may include genomic copy number variation, epigenetic status changes or transcriptional activation/repression.

Like many other cancers, genomic instability features significantly in HCC [264]. As many as 50 % miRNA genes are located in cancer-associated genomic regions or fragile sites [39]. Therefore, genomic DNA copy number variation represents a potentially important mechanism to regulate miRNA expression in HCC. Recent studies have provided evidence for DNA copy number variation at miRNA genomic loci in ovarian cancer, breast cancer and melanoma [265] and genomic amplification at Chr13q31 was reported to be responsible for miR-17-92 up-regulation in malignant lymphoma patients [266]. Members of miR-17-92 cluster were also found to be up-regulated in our profiling for HCC patients and genomic amplification may also be the reason behind our observation. Ding *et al.* reported genomic amplifications or deletions at miRNA loci of 22 out of the 129 miRNAs screened in HCC patient tissues. 75 % of these 22 miRNAs (16 miRNAs) showed concordant changes in expression [267]. Three of these miRNAs (miR-21, miR-151 and

miR-423) were reported to be associated with genomic amplification at the respective loci by up to 56 % and these miRNAs were also observed to be up-regulated in our study. Hence, copy number variation at the miRNA genomic loci may represent a common mechanism for miRNA deregulation in human cancers including HCC.

Epigenetic changes represent another mechanism for miRNA regulation. DNA methylation and histone modification can result in chromosomal remodeling which activates or represses transcription. Datta *et al*. reported differential miRNA expression in HCC cells after treatment with DNA methylation inhibitor (5-azacytidine) and histone deacetylase inhibitor (trichostatin A) and miR-1-1 is silenced through promoter DNA methylation in HCC patient samples [268]. miR-34a, which was up-regulated in tumors of HCC in our study, was shown to be up-regulated upon trichostatin A treatment, suggesting the involvement of histone acetylation in regulating miR-34a. Additional evidence has been provided by Furuta *et al.* who have demonstrated that miR-124 and miR-203 are epigenetically silenced through promoter methylation in HCC [269]. As epigenetic modification is heavily implicated in HCC [270], epigenetic changes may account for some of the aberrantly expressed miRNAs identified in HCC patients from our study. Further studies on epigenetic regulation of miRNAs, especially on the role of histone modification, will improve our understanding of this critical mechanism of miRNA regulation. Since epigenetic changes can be reversed with drug treatment, it might provide a novel therapy for HCC treatment.

Similar to their gene counterparts, miRNAs are tightly regulated at the level of transcription. V-myc myelocytomatosis viral oncogene homolog

(MYC) oncogene has been shown to transcriptionally regulate a number of cellular miRNAs. MYC positively regulates miR-17-92 cluster [271] and miR-9 [272] and negatively regulates miR-29a/b [273]. This may partly explain the aberrant pattern of such miRNA expression in HCC where MYC is frequently over-activated. Regulation of miRNA expression by another important cellular transcription factor nuclear factor kappa-B (NF-κB) has been reported for let-7i [274], miR-29a/b [273] and miR-143 [275], all of which were differentially expressed in HCC. In addition, miRNAs are also under the regulation of cellular signaling pathways. Tumor growth factor beta (TGF-β) signaling pathway is implicated in up-regulation of miR-224 in mouse granulosa cells [240] and miR-181b in HCC [276] while miR-34c is regulated by MAPK signaling pathway [277]. As more data emerges on transcriptional regulation of miRNAs, we can then better integrate miRNAs into existing cellular pathways that are dominated by protein-coding genes and further understand its role in aberrant miRNA expression in human cancer.

Hence, aberrantly expressed miRNAs in HCC may be due to one or more of the interdependent mechanisms mentioned above. Much effort is needed to understand the exact mechanisms of these observed miRNA deregulation in HCC which will in turn offer valuable insights into their possible therapeutic intervention to revert the functional impact of their deregulation in liver cells.

4.2 miRNA deregulation impacts on important cellular pathways

The identification of miRNA deregulation is only the first step to comprehend the role of miRNA in contributing to hepatocarcinogenesis. When

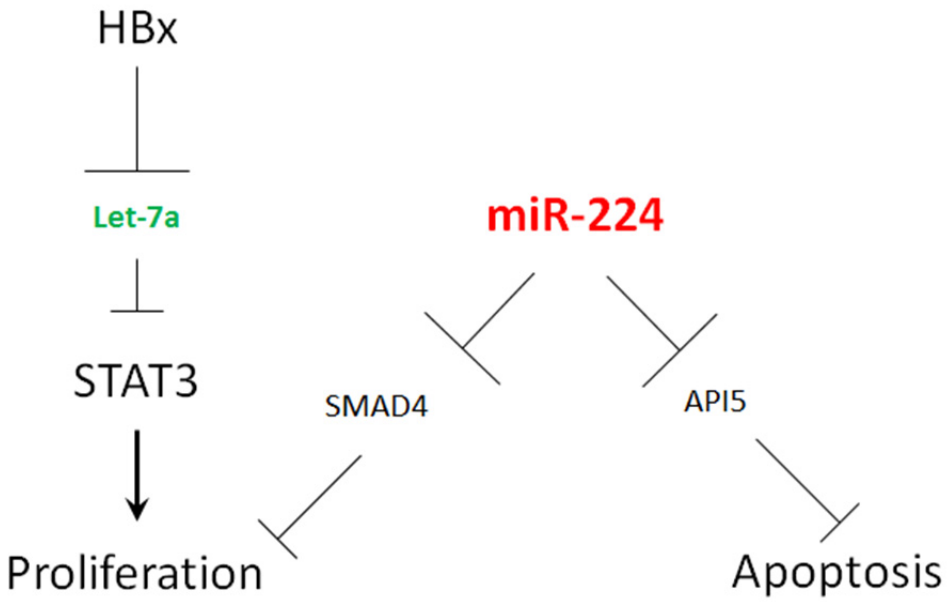

Figure 4.2.1 **Schematic diagram illustrating the deregulated miRNAs impacting on important cellular processes**. HBx mediated down-regulation of let-7a increases cell proliferation via releasing the inhibition on STAT3. miR-224 up-regulation increases cell proliferation via targeting SMAD4 and increases cell apoptosis via targeting API5.

our project started, the functional relevance of such miRNA deregulation with respect to their roles in deregulating crucial cellular pathways remains largely unknown and the relevant cellular targets of these deregulated miRNAs remain even more elusive. In Section 3.2 and 3.3 in Results, we have functionally characterized let-7 (significantly down-regulated miRNAs) and miR-224 (significantly up-regulated miRNA) to gain an understanding of the role of their deregulation in HCC.

4.2.1 HBx-Let-7-STAT3 pathway in HCC

Hepatitis B virus protein X (HBx) plays an important role in hepatocellular carcinogenesis. Our current understanding of the role of HBx focuses on its ability to deregulate cellular gene expression at the transcription level through its interaction with cellular factors to affect either cellular signal transduction pathways or gene transcription. In this study, we provided evidence that HBx can also deregulate cellular gene expression at the post-transcriptional level through deregulating cellular miRNAs such as let-7. It is not uncommon for viruses to evolve mechanisms to suppress RNA interference, which is an important antiviral defense. HIV-1 was recently reported to globally suppress host microRNA expression [278]. The Epstein-Barr virus latent membrane protein 1 was reported to activate miRNA-155 transcription [279]. This is the first study to demonstrate that the hepatitis B viral protein, HBx also plays a role in deregulating cellular miRNAs.

Let-7, the first miRNA identified in humans, was reported to target a number of important cellular oncogenes such as MYC[76], RAS[75] and HMGA2[280]. In this study, we identified and experimentally validated

another direct cellular target of let-7, STAT3, which is an important member of the JAK/STAT pathway. STAT3 was reported to be involved in many cellular processes including cell growth, survival, metastasis, angiogenesis and immune suppression, all of which favor tumor formation and progression [281]. STAT3 was also known to play critical role in liver regeneration and over-activation of STAT3 may play a role in contributing to HCC [282, 283]. In fact, constitutive activation of STAT3 alone is sufficient to transform normal mouse fibroblast [284]. Hence, STAT3 expression and activity are highly regulated via different mechanisms at different levels under physiological conditions. Mechanisms of STAT3 inhibition includes inhibition of Jak kinase activity through the activation of SOCS1 and SOCS3, also known as suppressor of cytokine signaling proteins [285] and protein inhibitors of activated STAT3 (PIAS) [286], or through the modulation of specific phosphatases such as protein phosphatase 2A (PP2A) [287]. The identification that let-7 microRNAs targets STAT3 reveals an additional level of regulation that STAT3 is subjected to, whereby modulation of its transcript/protein abundance is carried out by microRNAs. miRanda predicted a total of 122 putative miRNA binding sites along the 3'UTR of STAT3 (data not shown) suggesting that the STAT3 may be highly regulated not only at several different levels mentioned above but also at the post-transcriptional /translational level by several different miRNAs including let-7. Over-activation of STAT3 is frequently observed in solid tumors including breast, ovary and prostate tumors [281] where let-7 is also frequently under-represented [138]. However, STAT3 seems to be only one of several let-7 cellular targets through which let-7 acts to negatively regulate cell

proliferation since completely inhibiting STAT3 alone does not completely inhibit cell proliferation (Figure 3.2.5.(3)). This is not surprising as let-7 was also reported to target MYC [76] and RAS [41], both of which are important factors involved in cell proliferation. The fact that let-7 can target MYC, RAS, STAT3 and probably other proliferation factors yet to be discovered further demonstrates the role of let-7 as a master regulator of cell proliferation, whose loss of function will result in the deregulation of crucial cellular processes, leading to tumorigenesis. Unfortunately, we could not observe a statistically significant correlation between let-7a and STAT3 expression in the 20 HCC patients we examined (data not shown). This might be due to the fact that STAT3 regulation is very complex and additional HCC patient samples may need to be examined to better stratify the samples and reveal any potential correlation.

Although the down-regulation of let-7 promoted cell proliferation (Figure 3.2.4) and HBx was found to down-regulate let-7 expression (Figure 3.2.6.(1)), HBx expressing cells does not seem to show increased cell proliferation compared to control cells under normal conditions (Figure 3.2.6.(2)). This is probably due to the fact that HBx is also known to promote cell apoptosis in HepG2 cells [288]. To dissect the effect of HBx on cell proliferation from cell death, we proceeded to inhibit cell death by treating the cells with a general caspase inhibitor. When cell apoptosis is inhibited, HBx cells showed significantly higher cell proliferation than the control cells (Figure 3.2.6.(3)). Thus, our results strongly suggest that HBx can concurrently impact on both cell death and cell proliferation and HBx-mediated let-7 down-regulation functions to support cell proliferation in HBx

cells. These data are consistent with previous reports that HBx is a pleiotropic viral protein involved in both cell proliferation [259] and death [251]. In the multi-stage, multi-hit process of tumorigenesis, HBx may function as an opportunistic factor that can potentially help to promote tumor formation partially through its down-regulation of let-7 miRNAs in those cells where cell apoptosis are impaired. This could perhaps explain why different research groups observe different phenotypes in HBx-expressing cells [251, 259, 288] and mice [289, 290] since the predominant phenotype of HBx depends on other factors that make up the microenvironment in which the function of HBx is evaluated. Our study focuses on the role of full length HBx protein while Zhang et al recently demonstrated that a natural mutant form of carboxyl terminal truncated HBx may have a different effect on cellular proliferation [291]. This raises the possibility that HBx protein may have different functional domains to execute its pleiotropic effect on multiple cellular functions.

In summary, our work is the first report highlighting the role of the hepatitis viral protein HBx, in regulating cellular microRNAs expression. The HBx-let-7-STAT3 pathway identified in our study represents a clinically relevant pathway in which the pleiotropic HBx viral protein contributes to HCC and offers a new perspective in understanding the complexity of HCC.

4.2.2 miR-224 up-regulation in HCC impacts on multiple pathways

In addition to HBx mediated let-7 down-regulation, we also functionally characterized miR-224 up-regulation in HCC in our study. As shown in Figure 3.3.1, miR-224 expression is kept at a very low level in

normal liver samples, probably due to the fact that miR-224 resides in ChrXq28 where transcription is quiescent. miR-224 expression is significantly elevated in the non-tumor tissues from HCC patients and further increased in HCC tumor tissues. The fact that miR-224 expression increases as liver disease progresses makes it a possible biomarker for liver status. Further work is needed to evaluate the feasibility of miR-224 as a liver biomarker.

Functional characterization of miR-224 reveals that miR-224 is capable of increasing both cell proliferation and apoptosis through targeting different cellular genes (Figure 3.3.3 and Figure 3.3.4). miR-224 increases cell proliferation by targeting SMAD4, the central mediator of TGFβ pathway. TGFβ is an important protein regulating cell proliferation and cell differentiation by acting as an anti-proliferative factor. SMAD4 functions to transduce the signal from activated TGFβ receptor by binding to the activated SMAD2/3 and facilitating the translocation of the activated complex into the nucleus where anti-proliferation genes such as p21 and p15 are activated [292]. A number of tumor types have been reported to be associated with SMAD4 knockout mouse models [292], implicating dysfunctional TGFβ/SMAD pathway in carcinogenesis. We have demonstrated that miR-224 is able to interact with the putative miR-224 binding sites on SMAD4 3'UTR to negatively regulate the endogenous level of SMAD4 (Figure 3.3.8), promoting cell proliferation by possibly controlling TGFβ induced growth arrest. Hence, our data supported Yang *et al*'s hypothesis that miRNAs play roles in TGF-β/SMAD-pathway-induced tumor-suppressive effects [292].

Over-expression of miR-224 also sensitizes cells to undergo apoptosis by targeting API5, an apoptosis inhibitor. API5 was previously reported to act

downstream E2F and inhibit E2F-dependent apoptosis without affecting E2F-dependent transcription [293]. The observation that miR-224 is over-expressed in the tumors of HCC patients and that it plays a role in sensitizing cells to apoptosis via the inhibition of API5 expression seems to contradict conventional wisdom that apoptosis is reduced during carcinogenesis. Nonetheless, similar to miR-224, oncoproteins such as MYC and E1A, were found to sensitize cells to apoptosis upon minor insults that normal cells can usually resist e.g. serum depletion, DNA damaging agents, hypoxia, etc [294, 295]. Oncogenic changes that promote apoptosis are thought to provide the selective pressure for cells to override apoptosis during the multistage process of carcinogenesis [294] resulting in a resistant population of cells that accumulate heritable genetic mutations [296] during its increased lifespan thus facilitating oncogenic transformation. The dual role of miR-224 to influence both cell proliferation and cell death simultaneously may thus potentially hasten this selection process favoring cells that accumulate sufficient heritable genetic mutations to override apoptosis during the multistage of carcinogenesis. Sassen *et al.* has previously proposed that a single miRNA can potentially regulate opposing cellular activities such as cell proliferation and apoptosis [297] and miR-224 represents the first such miRNA identified.

4.2.3 miRNA network forms part of the HCC microenvironment

The main challenge to understand miRNA function is to identify relevant cellular targets through which these miRNAs act to regulate cellular processes. In this study, we have contributed to the understanding of let-7 in negatively regulating cell proliferation by the identification of STAT3 as a let-7 specific target. In addition, we have also identified API5 and SMAD4 as

relevant direct targets through which miR-224 positively regulate apoptosis and cell proliferation, respectively. However, we may be presenting only the tip of the iceberg as each miRNA is predicted to regulate hundreds of cellular targets. Indeed, let-7 is also known to regulate development [27] and miR-224 over-expression is also recently found to increase cell migration, invasion [240] and anchorage independent cell growth [298]. This highlights the fact that we are still far from complete understanding of even a single miRNA.

Moreover, we have adopted a common but simplified approach to study either let-7 down-regulation or miR-224 up-regulation in isolation. In reality, an HCC tumor with low let-7 expression is also frequently found to over-express miR-224. Taken our limited knowledge on let-7 and miR-224, we may speculate that the tumor cells with low let-7 and high miR-224 expression may be proliferating fast, less differentiated, more invasive and rapidly adapting to override apoptosis. The understanding of such complex network formed by multiple deregulated miRNAs is necessary to the role of such miRNA network in the real context of HCC. If we extrapolate this miRNA network from let-7 and miR-224 to the 66 significantly differentially expressed miRNAs in HCC, the exact combination of deregulated miRNAs in each of the HCC tumor samples may represent a unique signature of the specific tumor microenvironment associated with each individual HCC tumor, providing valuable information for personalized treatment. This hypothesis would require system biology approach to integrate a large dataset which comprises data from collective effort to characterize each miRNA.

4.3 miRNAs are potential diagnostic and therapeutic targets for HCC.

Current classification of HCC replies exclusively to clinical parameters and miRNA profile can be used to improve our ability to classify HCC and stratify prognosis. MiRNA expression signatures have been shown to be able to differentiate liver samples based on histology (hepatocellular adenoma from HCC), etiology (alcohol induced HCC from HBV associated HCC) and cancer gene mutation (β-catenin and hepatocyte nuclear factor 1α) [131]. Recently, a signature of 20 miRNAs has been shown to associate with HCC metastasis and correlates with overall survival [242]. Furthermore, high miR-221 levels in HCC were associated with tumor multifocality and reduced time to recurrence after surgery [226] and high miR-222 level negatively correlates with disease free survival [137]. MiRNA profiling, in combination with existing clinical parameters, may provide additional information on HCC classification and prognostic risk stratification and help oncologists make better informed decisions.

The easily detectable, serum bound miRNAs are good candidates to screen for specific biomarkers to improve existing screening strategies for early HCC detection. Significant differences in blood miRNA expression can be detected and associated with well-controlled liver tumorigenesis in a mouse model [243]. In human HCC, miR-500 is reported to be elevated in HCC patients and returned to normal after surgical resection [244]. MiR-500 and others represents potential diagnostic markers for HCC detection.

As miRNAs play an important role in HCC, they represent promising therapeutic targets for novel treatment strategies for HCC patients. Exciting data have emerged in the use of antagomirs which are chemically modified oligonucleotides to specifically and effectively knock down miRNA expression. Systemic delivery of antagomirs against miR-122 has resulted remarkably specific and stable down-regulation of miR-122 in the liver up to 20 days [245]. As miR-122 promotes HCV replication, knock-down of miR-122 may provide novel approach to manage HCV load in HCV-associated HCC. Recently Kota et al demonstrated the efficacy of the systemic delivery of miR-26a in an adeno-associated virus vector to protect disease progression without toxicity in a mouse liver cancer model [246], opening new door to miRNA replacement therapy.

4.4 Conclusions and perspectives

In conclusion, we identified a miRNA signature consisting of 66 significantly differentially expressed miRNAs between HCC tumors versus paired adjacent non-tumor tissues from 100 HCC patients. We subsequently functionally characterized let-7a down-regulation and miR-224 up-regulation. We identified that HBx is responsible for reduced let-7a expression and STAT3 as a direct cellular target through which let-7a negatively regulates cell proliferation. HBx-let-7-STAT3 pathway supports cell proliferation in HBx-expressing cells. On the other hand, miR-224 over-expression increases cell proliferation by targeting SMAD4 and sensitizes cells to apoptotic cell death by targeting API5.

We believe that the study of miRNA deregulation in HCC will continue to grow in the characterization of the various deregulated miRNAs in terms of the impact on various cellular functions and target identification. Our study concentrates on the HBV-associated HCC as our patient samples are mostly HBV positive. Similar studies should also be performed on other types of HCC with different etiology (e.g. HCV associated or alcohol induced HCC) to provide a complete picture of miRNA deregulation in HCC. Integrating individual miRNA to form complex miRNA regulatory networks will further improve our understanding of the role of miRNA deregulation in HCC and facilitate novel treatment strategies. The understanding of the exact mechanisms for crucial miRNA deregulations will also provide clues for its therapeutic intervention. We hope, in the near future, that we can better comprehend the complex nature of HCC from the miRNA perspective. We can integrate this new information with the existing knowledge of HCC to improve the clinical outcome of HCC patients.

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Appendix A

PUBLICATIONS

During the course of this project, a total of three papers, which are based on the work presented in this thesis, have been published in reputable peer-reviewed journals. They are listed here for reference.

Wang Y, Lee AT, Ma JZ, *et al*. *Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target.* **J Biol Chem**, 2008. 283(19): p. 13205-15.

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Wang Y, Caroline Lee GL. *Role of miR-224 in HCC: a tool for possible therapeutic intervention?* **Epigenomics** 2011 3(2): p. 235-243.

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Profiling MicroRNA Expression in Hepatocellular Carcinoma Reveals MicroRNA-224 Up-regulation and Apoptosis Inhibitor-5 as a MicroRNA-224-specific Target*[S]

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Like other cancers, aberrant gene regulation features significantly in hepatocellular carcinoma (HCC). MicroRNAs (miRNAs) were recently found to regulate gene expression at the post-transcriptional/translational levels. The expression profiles of 157 miRNAs were examined in 19 HCC patients, and 19 up-regulated and 3 down-regulated miRNAs were found to be associated with HCC. Putative gene targets of these 22 miRNAs were predicted *in silico* and were significantly enriched in 34 biological pathways, most of which are frequently dysregulated during carcinogenesis. Further characterization of microRNA-224 (miR-224), the most significantly up-regulated miRNA in HCC patients, revealed that miR-224 increases apoptotic cell death as well as proliferation and targets apoptosis inhibitor-5 (*API-5*) to inhibit *API-5* transcript expression. Significantly, miR-224 expression was found to be inversely correlated with *API-5* expression in HCC patients ($p < 0.05$). Hence, our findings define a true *in vivo* target of miR-224 and reaffirm the important role of miRNAs in the dysregulation of cellular processes that may ultimately lead to tumorigenesis.

Hepatocellular carcinoma (HCC)[5] is among the top 10 most prevalent cancers worldwide (1), accounting for ~600,000 deaths annually (2). The overall 5-year survival rate for HCC patients is less than 5% (3). Chemotherapeutic interventions are ineffective, and surgical resection/liver transplantation is the only treatment modality to confer survival benefit in HCC patients. Late clinical presentations have also led to poor prognosis for HCC patients. It is thus necessary to elucidate the molecular mechanisms underlying HCC and identify novel therapeutic targets and biomarkers for the early detection of HCC.

Like other cancers, aberrant gene regulation features significantly in HCC. Several reports on gene expression profiling of HCC patients have identified numerous pathways (*e.g.* proliferation, cell cycle regulation, apoptosis, angiogenesis, etc.) that may be dysregulated during hepatocarcinogenesis (see review in Ref. 4). Recently, an increasing number of reports have described a new class of small noncoding RNAs that are implicated in the regulation of gene expression at the post-transcriptional and translational level. These regulators are termed microRNAs (miRNAs), and their dysregulation may have implications in carcinogenesis.

miRNAs represent a class of noncoding RNAs whose processed products are ~22 nucleotides in length and regulate gene expression in plants and animals (5). To date, more than 500 miRNAs are predicted to be expressed in humans (6, 7). These miRNAs are estimated to regulate the expression of >5000 human genes or ~30% of all human proteins (8). It is likely that the interaction between miRNAs and their numerous mRNA targets may represent an important level of gene regulatory control in the cell (5).

The importance of miRNAs in cancer is highlighted by the observation that ~50% of miRNA genes are located in cancer-associated genomic regions or fragile sites (9–11). Importantly, miRNA expression is frequently dysregulated in several cancers including B-cell chronic lymphocytic leukemia (9, 12), Burkitt lymphoma (13), colorectal cancer (14), lung cancer (15, 16), and hepatocellular carcinoma (17). Additionally, differential expression of miRNAs have been found to be associated with post-operative survival in lung cancer patients (15) and are diagnostic and prognostic markers of lung cancer (16). miRNAs have been implicated to play both tumor suppressor and oncogenic roles (18).

Although much is known about the profiles of miRNAs in the various tissues/developmental stages, embryonic stem cell dif-

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[5] The abbreviations used are: HCC, hepatocellular carcinoma; miRNA, microRNA; RQ, relative quantity; GO, gene ontology; BrdUrd, bromodeoxyuridine; UTR, untranslated region; β-gal, β-galactosidase; MRP1, multidrug resistance-associated protein 1; EGFP, enhanced green fluorescence protein; PE, phycoerythrin.

ferentiation, brain development, hematopoietic lineage differentiation, as well as their deregulation in various cancers, less is known about the function of each of these miRNAs or their true cellular targets. Numerous algorithms have been developed to predict the putative cellular targets of these miRNAs including PicTar (19), miRBase Targets (7), and TargetScan (8). Some of the cellular targets that have been experimentally validated for the various miRNAs are HoxB8 (miR-196) (20), Hand2 (miR-1) (21), E2F1 (miR-17–5p and miR-20a) (22), Hox-A11 (miR-181) (23), LATS2 (miR-372/3) (24), Rb1 (miR-106a), TGFBR2 (miR-20a) (25), as well as IRAK1 and TRAF6 (miR-146) (26).

Here, we characterized the expression profiles of 157 miRNAs in 19 HCC patients and identified a set of significantly differentially expressed miRNAs associated with HCC. We also functionally characterized one of the differentially expressed miRNAs and identified its gene target.

EXPERIMENTAL PROCEDURES

Samples—Paired tumorous and adjacent nontumorous liver tissues from 19 hepatocellular carcinoma patients were obtained from the National Cancer Centre of the Singapore Tissue Repository with prior approval from the National Cancer Centre Institutional Review Board.

miRNA Extraction and Expression Profiling—The MirVana^{TM} miRNA isolation kit (Ambion, Austin, TX) was used to isolate total RNA including low molecular weight RNA from patient samples and cell lines according to the manufacturer's protocol. Expression of 157 verified human microRNAs was analyzed using the TaqMan MicroRNA assay human panel early access kit (Applied Biosystems), according to the manufacturer's instructions as previously described (27). Briefly, patient RNA samples were used as template for reverse transcription. Together with the high capacity cDNA archive kit, RNase inhibitors, and miRNA-specific reverse transcription primers (Applied Biosystems), the reverse transcription reactions were carried out in a 96-well plate format. Real time PCR was then performed with the reverse transcription products, TaqMan 2× Universal PCR Master Mix without UNG Amperase (Applied Biosystems), miRNA-specific TaqMan probes, and primers (Applied Biosystems) on an Applied Biosystems 7500 Fast Real Time PCR system with an initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (C_T) was then determined and defined as the fractional cycle number at which the fluorescence detected passes a fixed threshold. The Applied Biosystems 7500 Fast software was used to analyze the C_T values of different miRNAs normalized to an endogenous control (let-7a or U6). The normalized values (dC_T) from tumorous tissue were then compared with its paired nontumorous tissue, yielding miRNA differential expression profiles.

In Silico Analyses of miRNA Expression Data, Identification of Putative miRNA Targets, and Categorization of Biological Processes of These Putative miRNA Targets—Relative quantitation of the expression of miRNA was determined using the $2^{-\Delta\Delta C_t}$ method (28), and the results were expressed as $\log_2$ of the relative quantity (RQ) of the target miRNA normalized against hsa-let-7a ($\log_2$RQ). Differentially expressed miRNAs were identified by significant analysis of microarrays (29), with the false discovery rate threshold set at <5%. Clustering and visualization of the normalized data were performed with Cluster and TreeView (30), using average linkage and Pearson's correlation as a measurement for similarity. Computational identification of the putative miRNA targets was performed using PicTar (19), miRBase Targets Version 3.0 (7), and TargetScan Release 3.0 (8). A gene was considered to be a putative target of a given miRNA only if it was predicted by at least two of the three methods. Categorization of the biological processes of the putative miRNA gene target was performed with gene ontology (GO) using the Database for Annotation, Visualization, and Integrated Discovery GoCharts module (31), at level 5 annotations.

Validation of the Expression of miR-224 in Tumors of HCC Patients—Northern blot analysis was performed to validate miR-224 overexpression in the tumors of HCC patients. Briefly, 1 μg of total RNA (HCT116 cells transfected with Pre-miR^{TM} miR-224 precursor) or 2 μg of low molecular weight RNA from both the tumor and adjacent nontumorous tissues of a few of the same HCC patients that were previously profiled was separated on a 15% denaturing polyacrylamide gel and electroblotted onto a nylon membrane (Schleicher & Schuell GmbH; Dassel, Germany) at 300 mA for 30 min. The miRNA-224 probe (5′-TAAACGGAACCACTAGTGACTTG-3′ (17)) and U6-probe (5′-ATGTGCTGCCGAAGCGAGCAC-3′) were end-labeled with Redivue [γ-^{32}P]ATP (Amersham Biosciences) using T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA) and purified using the nucleotide removal kit according to the manufacturer's instructions (Qiagen). Hybridization was performed using Express Hybridization solution (Clontech, Mountain View, CA) at 42 °C for 16 h, and the blots were exposed to Hyperfilm MP (Amersham Biosciences).

Growth and Viability of HCT116 Cells Transfected with miR-224—The human colon cancer cell line, HCT116, was grown in McCoy's medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2. To determine the effect of miR-224 on growth and viability properties of HCT116 cells, 4×10^5 cells were transfected with either 30 nM of Pre-miR^{TM} miR-224 precursor molecule (miR-224 precursor) or 30 nM of Pre-miR^{TM} miRNA Precursor Molecules-Negative Control 1 (Control) (AM17110) (Ambion) using siPORT^{TM} amine transfection agent (Ambion) following the manufacturer's protocol. The time when transfection commenced was considered as time 0. Sixteen hours after incubation in medium containing siPORT^{TM} amine transfection agent, these transfected cells were transferred into normal growth medium. Viable cells were determined through trypan blue dye exclusion. The growth properties of these cells were expressed as percentages of viable cells at the respective time points relative to time 0. Viability of these cells was expressed as the percentages of viable cells relative to the total number of cells (both dead and alive) at each individual time point. The results were obtained by counting cells from the same experiment twice in three independent experiments.

Apoptotic and Cell Proliferation Properties of HCT116 Cells Transfected with miR-224—HCT116 cells were transfected using siPORT^{TM} amine transfection agent with either 60 nM control or 30 nM miR-224 precursor and 30 nM control or 30 nM

miR-224 precursor and 30 nM anti-miRTM miR-224 inhibitor (miR-224 inhibitor; AM17000, Ambion). Apoptosis assay was performed 48 h post-transfection using the Annexin V-PE apoptosis detection kit I (BD Biosciences) according to the manufacturer's protocols and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Apoptotic cells were represented by high PE-Annexin V and low 7-amino-actinomycin fluorescence signals. Cell proliferation assays were performed 24 h post-transfection using the BrdUrd cell proliferation assay kit (Calbiochem, San Diego, CA) following the manufacturer's protocol. BrdUrd incorporation measured as absorbance at A_{450} in a SpectroMAX PLUS microplate reader (Molecular Devices, Sunnyvale, CA) 48 h after it was added to the cells.

Apoptotic Properties of Primary Liver Cell Line Immortalized with SV40 T-antigen, THLE-3, Transfected with miR-224 Inhibitor—To evaluate whether miR-224 influence the apoptotic potential of a primary liver cell line transformed with SV40 T-antigen, THLE-3 cells were transfected in collagen-coated plates with either 60 nM control or 60 nM miR-224 inhibitor using siPORT^{TM} amine transfection agent. The transfected cells were then treated with ~20 J/m^2 UV 24 h post-transfection, and apoptosis assay was performed 48 h after transfection.

Generation of the miR-224 Target, API-5 3′-UTR-Reporter Construct—To experimentally validate whether the *API-5* gene is an *in vivo* target of miR-224, the 3′-UTR of the *API-5* gene was amplified from nontumorous human liver tissue using primers API5primerF1 and API5primerR1 as shown in supplemental Fig. S1. The 3′-UTR was then cloned downstream to a β-galactosidase (β-gal) reporter gene driven by the human multidrug resistance-associated protein 1 (MRP1) promoter in a construct that also contained the enhanced green fluorescence protein (EGFP) gene for normalization of transfection efficiency (32) (see Fig. 3*B*). The human MRP1 promoter was chosen over the constitutive human cytomegalovirus promoter, because the MRP1 promoter is ~30 times weaker than the cytomegalovirus promoter (data not shown), which will facilitate the measurement of subtler changes in reporter gene activity. A mutant pAPI5-3UTR-MUT was also generated by PCR mutagenesis using primers as shown in supplemental Fig. S1. Three point mutations were generated on each of the three miR-224 target recognition sites/seed as shown in Fig. 3*C*. These mutant recognition sites were verified *in silico* not to bind to any of the known human miRNAs using miRBase (Release 8.1, May 2006). The mutant construct generated was confirmed by sequencing.

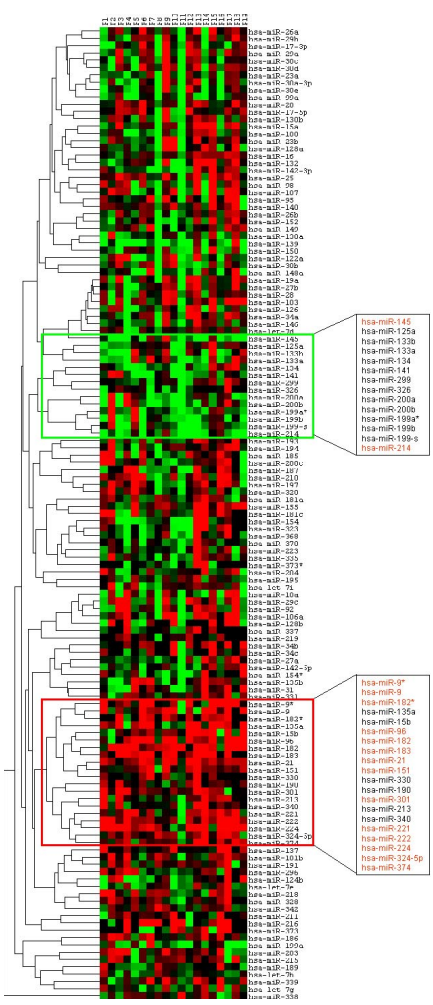
Characterization of the Effect of miR224 on API-5 3′-UTR-Reporter Construct—HCT116 cells were transfected in 6-well plates by using siPORT^{TM} amine transfection agent (Ambion, Austin, TX) according to the manufacturer's instructions with either 1.0 μg of the β-gal reporter construct containing the wild type 2035-bp 3′-UTR sequence of human *API-5* (termed pAPI5-3UTR-WT) or β-gal reporter construct containing the mutant 3′-UTR sequence of human *API-5* (termed pAPI5-3UTR-MUT) and co-transfected with either 30 nM of miR-224 precursor (Ambion) or 30 nM of control (Ambion). β-Gal reporter gene activity was assayed kinetically using chlorophenol red-β-D-galactopyranoside as substrate and measured at 1-min intervals over 60 min at 570 nm in a SpectraMAX PLUS microplate reader (Molecular Devices) with crude lysate from the transfected cells harvested 24 h post-transfection. To normalize for differences in transfection efficiencies, Western blot analyses were performed using 0.02 μg/ml mouse anti-EGFP (Roche Applied Science) and 1:100,000 horseradish peroxidase-conjugated goat anti-mouse (Pierce) secondary antibodies. β-Gal activity was then normalized against EGFP expression levels. The data was also normalized against differences in basal β-gal activity when either the pAPI5-3UTR-WT or pAPI5-3UTR-MT construct, but not miR-224 precursor or Control, was transfected.

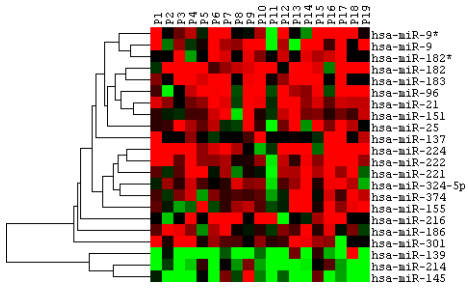
Quantitation of the API-5 mRNA Levels in Patient Samples and HCT116/THLE-3 Cells Transfected with miR-224—Reverse transcription real time PCR was performed to quantitate the *API-5* transcript levels in patient samples and transfected HCT116/THLE-3 cells. cDNA was synthesized from total RNA using a high capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. Real time PCR was performed in an Applied Biosystems 7500 real time PCR system using the QuantiTect^{TM} SYBR Green PCR kit (Qiagen). Amplification reactions included cDNA template (25 ng), *API-5* primers (forward, 5′-TAGTGGGTTTGGAGAAGTTC-3′; reverse, 5′-TCACTTGATAGGCATCTTTATG-3′) (0.25 pmol/μl), and 2× PCR Master Mix (5 μl; Qiagen) in a total volume of 10 μl. Amplification conditions include an initial denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. SYBR Green fluorescence was measured after each extension step.

Statistical Analysis of Experimental Data—Student's *t* test was performed to analyze the significance of differences between sample means obtained from at least three experiments.

RESULTS

miRNA Expression Profiling Identifies Dysregulation of miRNAs That Are Associated with HCC—In this study, the expression profiles of 157 mature miRNAs were determined in 19 HCC tumors and adjacent nontumorous liver tissues using the TaqMan MicroRNA assays human panel early access kit (Applied Biosystems) and normalized against hsa-let-7a as recommended by the manufacturer. Similar results were obtained when the same data set was also normalized against U6 RNA levels (data not shown) (27). Of the 157 miRNAs, 133 exhibited differential expression in at least 50% of the HCC patients, and these are presented in Fig. 1*A* as a TreeView Heat Map. From the TreeView Heat Map, there seem to be clusters of 20 miRNAs (*red box*) and 14 miRNAs (*green box*) that were up-regulated and down-regulated, respectively, in the tumors of HCC patients. The trends of differential expression of some of these miRNAs (miR-199a, miR-200a, miR-125a, and miR-224) were consistent with those observed in the only other previous report on miRNA expression in HCC (17). Significance analysis of microarrays (21) was then utilized to identify miRNAs that displayed significant differential expression between the tumor and adjacent nontumorous liver tissues of HCC patients (supplemental Fig. S2). When the false discovery rate was set to <5%, only 19 miRNAs were found to be significantly up-regulated, whereas three were determined to be significantly down-regulated, with the mean fold change of the most highly up-reg-





Significantly Up-regulated in Tumors (19)

miRNA	Mean fold change (log_2RQ)	No of predicted targets
hsa-miR-224	3.50	114
hsa-miR-182	3.46	447
hsa-miR-183	3.36	201
hsa-miR-96	2.64	498
hsa-miR-9*	2.61	46
hsa-miR-9	2.56	538
hsa-miR-222	2.32	107
hsa-miR-216	2.10	77
hsa-miR-21	2.07	125
hsa-miR-186	2.01	136
hsa-miR-301	1.59	315
hsa-miR-221	1.57	115
hsa-miR-155	1.44	151
hsa-miR-182*	1.44	12
hsa-miR-137	1.34	307
hsa-miR-25	1.32	229
hsa-miR-324-5p	1.28	32
hsa-miR-151	1.26	22
hsa-miR-374	1.23	133

Significantly Down-regulated in tumors (3)

miRNA	Mean fold change (log_2RQ)	No of predicted targets
hsa-miR-214	-2.43	159
hsa-miR-145	-2.64	147
hsa-miR-139	-3.26	173

FIGURE 1. **Profiles of miRNA expression in HCC patients.** miRNA expression was examined between the HCC tumor and adjacent nontumorous liver tissues from 19 HCC patients as described under "Experimental Procedures." *A*, hierarchical clustering of 133 miRNAs that are differentially expressed in the tumors of at least 50% of the 19 HCC patients. The different HCC patients are represented on the *x* axis, and the results are presented as the mean fold change in miRNA expression of tumor *versus* adjacent nontumorous tissue in each patient. *Red* represents miRNAs that are overexpressed in the tumors, whereas *green* represents miRNAs whose expression is down-regulated in tumors. The *green box* shows a cluster of miRNAs that are down-regulated in HCC tumors, whereas the *red box* display the cluster of miRNAs whose expression of are up-regulated in the tumor. *B*, *top panel*, hierarchical clustering of the 22 significantly up- and down-regulated miRNAs obtained using significance analysis of microarrays with false discovery rate of <5%. *Bottom left panel*, table of the 19 miRNAs that are significantly overexpressed in the tumors of HCC patients represented as mean fold change of tumor *versus* nontumorous tissue and expressed as log_2RQ. *Bottom right panel*, table of the three miRNAs that are significantly down-regulated in the tumors of HCC patients represented as mean fold change of tumor *versus* nontumorous tissue and expressed as log_2RQ.

ulated miRNA (miR-224) being $log_2RQ = 3.50$ and that for the most down-regulated miRNA (miR-139) being $log_2RQ = -3.26$ (Fig. 1*B*). Two of the three significantly down-regulated miRNAs (miR-145 and miR-214) reside in the cluster of down-regulated miRNAs (*green box*), whereas 14 of the 19 up-regulated miRNAs (*red box*) reside in the cluster of up-regulated miRNAs shown in Fig. 1*A*.

Majority of the Predicted Targets of These 22 Differentially Regulated miRNAs Reside in Pathways Reported to Be Dysregulated during Carcinogenesis—An *in silico* strategy was employed to obtain a glimpse of the potential roles of these 22 differentially expressed miRNAs in HCC carcinogenesis. Putative gene targets of all 22 miRNAs (Fig. 1*B*) were predicted using PicTar (19), miRBase Targets Version 3.0 (7), and TargetScan Release 3.0 (8), and only gene targets predicted by at least two of the three algorithms (supplemental Fig. S3) were further analyzed to reduce the chance of false positives (19, 34, 35). Between 12 and more than 500 gene targets were predicted for each of the 19 miRNAs that were significantly up-regulated in tumors of HCC patients (Fig. 1*B*, *bottom left panel*), whereas 147–173 gene targets were predicted for each of the three miRNAs that were significantly down-regulated (Fig. 1*B*, *bottom right panel*). The pathways that these predicted gene targets reside were annotated using GO (36) and the Database for Annotation, Visualization, and Integrated Discovery (GoCharts module) (31) to determine biological pathways, which were significantly over-represented. Based on a *p* value of 0.01, 48% (1148/2391) of the predicted nonoverlapping gene targets of the miRNAs resided within 35 biological pathways including several classical cancer-associated pathways like

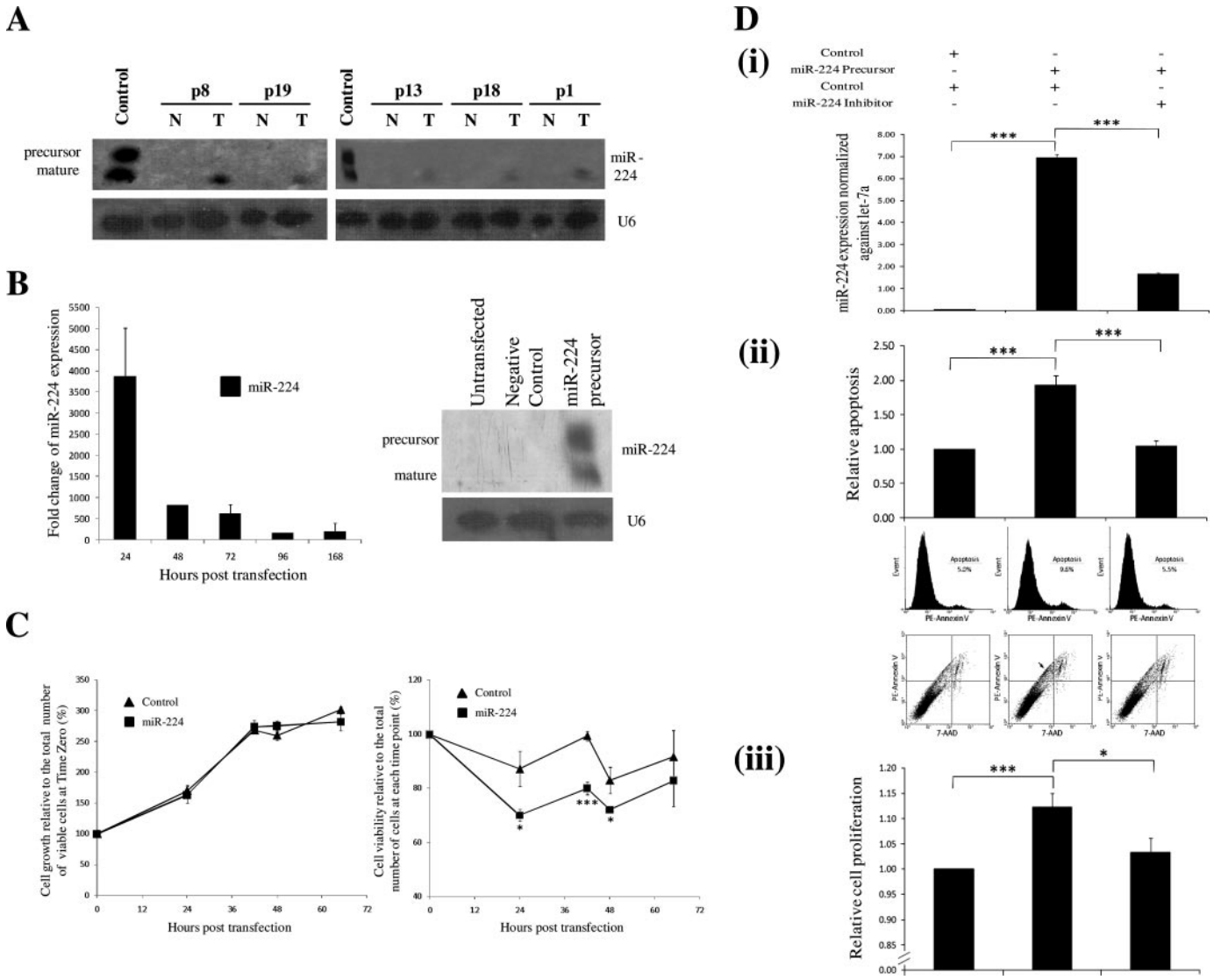


FIGURE 2. **Functional characterization of miR-224.** *A*, Northern blot analyses to validate the increased miR-224 expression in tumor tissues of representative HCC patients. *P(number)* represent the identity of the patients corresponding to the same patients in Fig. 1. *T* denotes tumor tissue from indicated HCC patients, whereas *N* denotes the paired nontumorous liver tissues. *Control* represent total RNA isolated from HCT116 cells 72 h after transfection with miR-224 precursor and hybridized with miR-224 probe. *B*, miR-224 expression in HCT116 cells. The *left panel* shows results from TaqMan MicroRNA individual assay for hsa-miR-224 (Applied Biosystems) in HCT-116 cells after transfection with miR-224 and normalization against hsa-Let-7a for the various time points. The results are expressed as the fold difference between miR-224 expression in miR-224 precursor transfected cells *versus* control transfected cells. The *right panel* shows Northern blot Analyses of untransfected HCT116 cells or HCT116 cells carrying control or miR-224 precursor, 72 h after transfection. *C*, *left panel*, cell growth expressed as percentage of viable cells at the respective time points relative to the transfection start time, time 0. miR-224 precursor transfected cells are represented by *squares*, and control transfected cells are represented by *triangles*. *Right panel*, viability of cells expressed as the percentage of viable cells relative to the total number of cells at a particular time point. The results were obtained by counting cells from the same experiment twice in three independent experiments. *D*, *panel i*, miRNA expression of cells transfected with control, miR-224 precursors, or miR-224 precursors and miR-224 inhibitors. *Panel ii*, relative apoptosis of similarly treated cells, as assayed using PE-conjugated annexin V staining. Apoptotic cells were detected as high in PE-annexin V staining and low in 7-amino-actinomycin (*7-AAD*) staining. The profiles shown are representative from three independent experiments. *Panel iii*, relative cell proliferation of similarly treated cells assayed using the BrdUrd proliferation assay kit. In *B–D*, data are from at least three independent experiments and shown as the means ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, denotes $p < 0.001$.

transcription, regulation of progression through cell cycle and Wnt receptor signaling pathway (supplemental Table S1). 80% of these 35 biological pathways have been reported to be implicated in the carcinogenesis process (supplemental Table S1), strongly suggesting that the miRNAs we observed to be differentially expressed in HCC patients target genes whose dysregulation may play significant roles in carcinogenesis.

miR-224 Overexpression Decreases Cell Viability and Sensitizes Cells to Apoptotic Cell Death—To gain further insights into how dysregulation of these miRNAs may play a role in carcinogenesis, we further characterized miR-224, the most up-regulated miRNA ($\log_2$RQ = 3.5 or fold change of 11.28) in our study and the only miRNA that was also reported in another study to be up-regulated in HCC patients. Northern blot analysis confirmed that miR-224 was up-regulated in the tumors of HCC patients (Fig. 2*A*). The functional significance of increased miR-224 expression in the cells was evaluated by transfecting miR-224 precursor into the human colorectal HCT116 cells, which exhibit low endogenous miR-224 expression (data not shown). miR-224 expression peaked 24 h post-

transfection, but its expression remained high (>100-fold higher than that of Control transfected cells) even at 168 h post-transfection (Fig. 2*B*). Cell growth, viability, and apoptotic profiles, some of the primary hallmarks of cancer (37), were examined on miR-224 expressing cells. Cell growth remained generally unaffected in cells transfected with either miR-224 precursor or Control (Fig. 2*C*, *left panel*), but cell viability was significantly reduced (up to 20% reduced cell viability; $p < 0.05$) in cells transfected with miR-224 precursor compared with cells transfected with Control (Fig. 2*C*, *right panel*). These data suggest that miR-224 seems to affect the viability rather than the growth of these cells.

Annexin V labeling was performed to delineate whether the reduced viability of cells overexpressing miR-224 is due to increased cell death through apoptosis. miR-224 precursor- or control-transfected HCT116 cells were harvested 48 h post-transfection, stained with PE-conjugated annexin V and 7-amino-actinomycin, and analyzed on the flow cytometer. Apoptotic cells are represented as cells with high PE-conjugated annexin V fluorescence signals and low 7-amino-actinomycin fluorescence signals. As shown in Fig. 2*D* (*panel ii*), ~2-fold greater apoptosis was observed in cells expressing miR-224 than in cells expressing the control precursors ($p < 0.001$). Significantly, when miR-224 inhibitor was introduced into cells overexpressing miR-224, the miR-224 expression was significantly reduced ($p < 0.001$) (Fig. 2*D*, *panel i*), and significantly less apoptosis ($p < 0.001$) (Fig. 2*D*, *panel ii*) was observed, consistent with cells not overexpressing miR-224. Hence, the reduced cell viability observed in miR-224-expressing cells is likely due to increased apoptosis of these cells in the presence of miR-224 as evident from the increased apoptosis observed in miR-224-overexpressing cells and the restoration of its normal phenotype when miR-224 inhibitor was also introduced.

miR-224 Overexpression Increases Cell Proliferation—Curiously, although miR-224 overexpression reduced cell viability (Fig. 2*C*, *right panel*) and increased apoptosis (Fig. 2*D*, *panel ii*), it does not seem to affect cell growth (Fig. 2*C*, *left panel*) as measured by the percentage of viable cells at each time point relative to time 0. We thus hypothesize that this observation could be due to miR-224, also increasing cell proliferation in addition to increasing cell apoptosis, thus resulting a similar number of viable cells in miR-224-expressing cells compared with control cells at any time point. The proliferation potential of these cells was then examined using the BrdUrd incorporation assay. As shown in Fig. 2*D* (*panel iii*), transfection of miR-224 precursor into these cells significantly increased the proliferation potential of these cells ($p < 0.001$), whereas the co-transfection of the miR-224 inhibitor with the miR-224 precursors restored the cell proliferation potential to normal.

miR-224 Targets API-5—To elucidate the gene target that miR-224 acts through to sensitize cells to apoptosis, we examined the putative targets of miR-224 more closely and short-listed seven (H3F3B, *API-5*, ARMCX2, NUP153, FOSB, TRIM9, and HOXD10) putative targets that ranked among the top five in at least one *in silico* target prediction program and is also identified by at least one other prediction program (Fig. 3*A*). These seven putative targets were found to be involved in several biological processes that are significantly over-represented. FOSB and HOXD10 function as transcriptional regulators. HOXD10 has also been implicated as a tumor suppressor (38) and inhibitor of angiogenesis (39), whereas *API-5* has been shown to be an apoptosis regulator (40–42). Other significant biological processes that involve these putative genes include chromosome organization for H3F3B (43), microtubule binding for TRIM9 (33), and intracellular/nuclear transport for NUP153 (44). Although the gene targets of miR-224 reside in varied biological pathways that are true for any miRNA (35), the pathways of nearly all of the top seven miR-224 gene targets are primarily those most associated with cancer, namely transcription, cell cycle, or apoptosis regulation and nucleus/chromosomal organization. Of these seven top miR-224 gene targets, *API-5* seems to be the most appropriate candidate through which miR-224 may act to increase apoptosis in cells because *API-5* has previously been reported to be an anti-apoptotic gene (40–42).

We proceeded to validate that *API-5* was in fact a true gene target of miR-224. Three miR-224-binding sites were identified along the 2035-bp-ylong 3′-UTR of *API-5* (miRBase release 8.1, May 2006) (Fig. 3*C*). The entire wild type 3′-UTR of *API-5* as well as a mutant form in which all three putative miR-224-binding sites were mutated was then cloned downstream the β-gal reporter gene (Fig. 3*B*). The specific interaction between miR-224 and *API-5* 3′-UTR to inhibit reporter gene activity was evident in Fig. 3*D*. When miR-224 precursor was co-transfected with wild type *API-5* 3′-UTR reporter construct, significantly reduced β-gal activity was observed compared with cells co-transfected with miR-224 precursor and mutant *API-5* 3′-UTR reporter construct ($p < 0.01$). No significant difference in β-gal activity was observed between HCT116 cells carrying either the wild type or mutant *API-5* 3′-UTR reporter construct when co-transfected with control precursor molecules (Fig. 3*D*).

To evaluate whether miR-224 can affect the endogenous expression of *API-5*, HCT116 cells were initially transfected with either miR-224 precursor or control. miR-224 inhibitor or control were then introduced into these transfected cells 24 h later, and reverse transcription real time PCR was performed at various time points after this second transfection. As shown in Fig. 3*E*, *API-5* expression was significantly lower in miR-224 precursor transfected HCT116 cells compared with the control cells ($p < 0.001$) across time points examined, consistent with previous reports that miRNAs can also down-regulate gene expression at the transcript level (45). When miR-224 inhibitor was transfected into miR-224-overexpressing cells, the *API-5* transcript levels significant increased ($p < 0.001$) at the two time points examined, suggesting that the miR-224 inhibitor was able to rescue the inhibition of *API-5* expression by miR-224. This observation that miR-224 decreased *API-5* expression corroborates with our earlier observation that miR-224 increases apoptotic cell death and strongly suggests that miR-224 exerts this effect through regulating the expression of *API-5*.

Inhibition of the Endogenous Expression of miR-224 Up-regulates API-5 Expression and Reduced Apoptotic Cell Death in the Immortalized Primary Liver Cell Line, THLE-3—Thus far, we observed that miR-224 is overexpressed in the majority of

A

Gene	Entrez Gene ID	Gene description	PicTar ranking	miRBase ranking	TargetScan ranking
H3F3B	3021	H3 histone, family 3B (H3.3B)	1	-	122
API-5	8539	Apoptosis inhibitor 5	2	-	99
ARMCX2	9823	Armadillo repeat containing, X-linked 2	4	198	-
NUP153	9972	nucleoporin 153kDa	168	1	46
FOSB	2354	FBJ murine osteosarcoma viral oncogene homolog B	222	4	125
TRIM9	114088	Tripartite Motif 9	12	174	1
HOXD10	3236	Homeobox D10	3	-	3

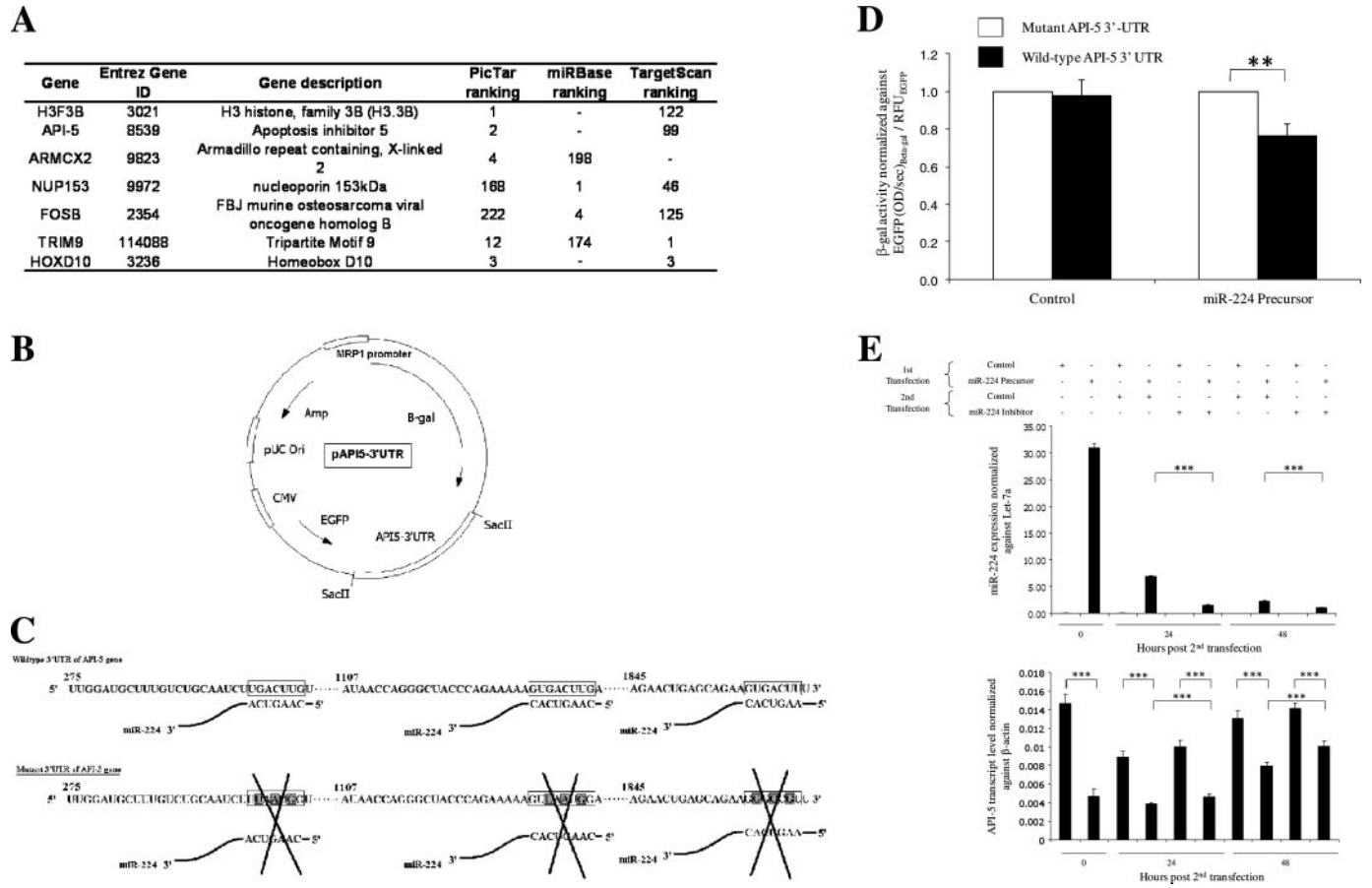


FIGURE 3. **Identification and characterization of a gene target of miR-224.** *A*, table showing the list of top seven putative targets of miR-224 determined *in silico* using PicTar, miRBase, or TargetScan algorithms. These targets are ranked among the top five in at least one algorithm and are listed as a putative target in at least one other algorithm. *B*, schematic diagram of the construct utilized to validate that the API-5 3′-UTR is target of miR-224. The β-gal reporter is driven by the MRP1 promoter (32). The *API*-5 3′-UTR (wild type or mutant) is cloned downstream the β-gal reporter. EGFP driven by the cytomegalovirus promoter is used to normalized for differences in transfection efficiencies. The construct also contain a pUC origin of replication (*Ori*) and an ampicillin resistance (*Amp*) for growth and selection in bacterial cells. *C*, schematic representation of the 3′-UTR of API-5 in which the predicted sequences of the three miR-224-binding sites is boxed. The bases *shaded gray* represent sequences that have been mutated to abolish miR-224 binding to the *API*-5 3′-UTR. Mutated sequences were verified *in silico* not to bind to miR-224 or any of the known human miRNAs. The mutant construct generated was confirmed by sequencing. *D*, action of miR-224 on the 3′-UTR of API-5 examined through normalized β-gal reporter activities in cells co-transfected with miR-224 precursors or control and wild type API-5 3′-UTR (*black bar*) or mutant API-5 3′-UTR (*white bar*) reporter constructs. The results show data from at least three independent experiments. *E*, miRNA expression (normalized against Let-7a) (*top panel*) and endogenous API-5 mRNA expression (normalized against β-actin) (*bottom panel*) in control or miR-224 precursor or miR-224 precursor and miR-224 inhibitor transfected cells. The cells were initially transfected with either control or miR-224 precursors. Twenty-four hours later, the same cells were transfected with either control or miR-224 inhibitors. miR-224 and *API*-5 RNA levels were measured at various time points after the second transfection. The results show data from at least three independent experiments. In *D* and *E*, the data are expressed as the means ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

HCC patients examined and demonstrated experimentally that the overexpression of miR-224 in HCT116 cells resulted increased apoptosis and reduced expression of the apoptosis inhibitor, *API-5* gene. We proceeded to evaluate the functional consequences of inhibiting the endogenous expression of miR-224 in an immortalized primary liver cell line, THLE-3. The introduction of miR-224 inhibitor into THLE-3 cells was found to significantly inhibit the endogenous expression of miR-224 in these cells ($p < 0.001$) (Fig. 4*A*, *top panel*) and significantly increased the expression of the *API*-5 gene (Fig. 4*A*, *bottom panel*). Inhibiting endogenous miR-224 expression in the THLE-3 primary liver cells was also found to protect these cells from UV-induced apoptotic cell death ($p < 0.05$) (Fig. 4*B*).

The Level of API-5 mRNA Expression Was Inversely Correlated with miR-224 Expression in 18 HCC Patients—We proceeded to examine whether there is any correlation between miR-224 and *API*-5 expression in HCC patients to evaluate the clinical significance of our observations. As evident in Fig. 6, a statistically significant inverse correlation was observed between miR-224 and *API*-5 expression in HCC patients (Pearson Coefficient $r = -0.471$, $R^2 = 0.221$ at $p < 0.05$).

DISCUSSION

MicroRNAs have recently been implicated to play important roles in cancers because >50% of miRNA genes reside in cancer-associated genomic regions, and their expression has been found to be dysregulated in various cancers. Thus far, only one study examined miRNA profiles in HCC patients, and they identified five miRNAs that were significantly down-regulated (miR-199a, miR-199a*, miR-200a, miR-125a, and miR195) and three that were significantly up-regulated (miR-224, miR-18, and miR-p18) (17). miR-18 and miR-p18 were not examined in this study, whereas miR-199a, miR-199a*, miR-200a, miR-125a, and miR-195 were also generally down-regulated in our study,

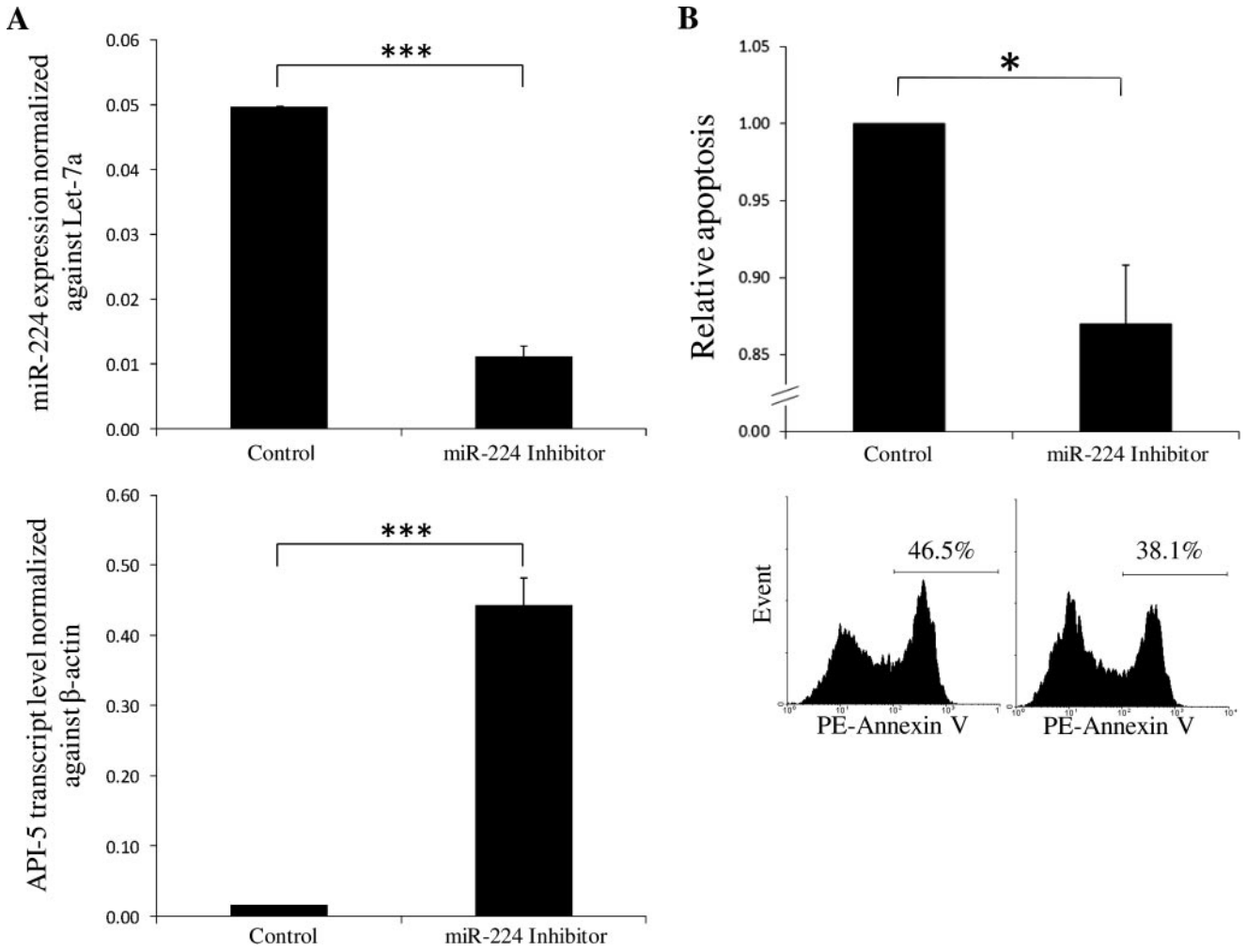


FIGURE 4. **Inhibiting endogenous miR-224 in THLE-3, an immortalized primary liver cell line, increased *API-5* expression and decreased number of apoptotic cells.** *A*, miR-224 (*top panel*) and *API-5* (*bottom panel*) expression in THLE-3 cells transfected with either control or miR-224 inhibitor. *B*, relative apoptosis of THLE-3 cells transfected with either control or miR-224 inhibitor. The results show data from at least three independent experiments, expressed as the means ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

although they were not statistically significant. Only miR-224 was also found to be significantly up-regulated in our study.

Our study identified 19 up-regulated and three down-regulated miRNAs that may be associated with HCC. Interestingly, four of the 19 significantly up-regulated miRNAs (miR-182, miR-182*, miR-183, and miR-96) reside together on chromosome 7q32.2 in the intergenic region between two protein coding genes, namely the transcription factor α palindrome-binding protein, α-pal (also known as nuclear respiratory factor 1) and the ubiquitin-conjugating enzyme, E2H. The coordinate up-regulation of these miRNAs that resides within the same cluster suggests that this region of chromosome 7 may be amplified. A search through the Comparative Genome Hybridization data base revealed that gain of chromosome 7 is commonly observed in HCC and other cancers including breast, prostate, colorectal, gastro-esophageal, lung and bronchial, head and neck, as well as aggressive lymphomas. Such coordinate expression of several miRNA and/or genes was also observed at the miR-17–92 cluster whose expression is up-regulated in lung cancer and plays a role in cell proliferation (46). However, further examination of nuclear respiratory factor 1 and E2H transcript expression in these HCC patients revealed that these genes were not significantly overexpressed in the tumors of these patients (data not shown).

Although our study found that more miRNAs are up-regulated in HCC patients, another study that examined 334 tissues from patients with different cancers found that there is a general trend of down-regulation of miRNA expression (47). Nonetheless, a recent study that examined 540 samples from patients with different cancers identified a solid tumor miRNA signature that primarily comprises up-regulated miRNAs (25).

Some of the miRNAs that we observed to be dysregulated in HCC patients exhibited similar trends of dysregulation in other cancers. miR-96 and miR-183 were also found to be up-regulated in colorectal cancer (27), whereas miR-21 and miR-155 were similarly overexpressed in breast, lung, and colon cancers (25). miR-224 was also up-regulated in HCC patients in another report (17), and miR-221 was also up-regulated in colon, pancreas, and stomach tumors (25). These observations suggest a common miRNA regulation pathway shared by different solid tumors.

The biological pathways that are affected through the dysregulation of these 22 miRNAs were further examined. Gene targets for all 22 miRNAs were predicted *in silico* and GO/Database for Annotation, Visualization, and Integrated Discovery was employed to functionally annotate these gene targets. We found that nearly 50% of the nonoverlapping gene targets reside within 34 biological processes. 80% of these 34 biological processes has been implicated in carcinogenesis (supplemental Table S1) including proliferation (GO terms: regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolism; regulation of progression through cell cycle; cell growth; positive regulation of cellular metabolism); cell death (GO term: apoptosis); metastasis (GO term: cell migration); and Wnt (48) and Notch (49) signaling pathways.

To gain a better understanding of how dysregulation of these miRNAs may play a role in carcinogenesis, we further characterized miR-224, the most up-regulated miRNA in our study. We observed that HCT116 cells overexpressing miR-224 exhibited similar rate of cell growth but significantly lower cell viability than control cells (Fig. 2*C*). This decrease in cell viability was found to be due to a significant increase of apoptosis in miR-224-expressing cells that can be reversed by the addition of miR-224 inhibitors (Fig. 2*D*, *panel ii*). Interestingly, miR-224-overexpressing cells also significantly increase the proliferation potential of the cells, which can be reversed by the addition of miR-224 inhibitors (Fig. 2*D*, *panel iii*). The ability of a single gene to be involved in both proliferation and apoptosis has been previously demonstrated (*e.g. myc*) (50).

To elucidate the gene target of miR-224 that may play a role in regulating apoptosis and hence account for the phenotype we observed, we shortlisted seven putative miR-224 gene targets that are ranked highly by at least one prediction program (Fig. 3*A*). Of these seven putative targets, apoptosis inhibitor 5 (*API-5*), also known as AAC-11, seems to be the most likely candidate because *API-5* was reported as an anti-apoptotic gene (40–42). Three potential miR-224-binding sites were identified at the 3′-UTR of *API-5* (Fig. 3*C*). Our results showed that miR-224 specifically interacted with the 3′-UTR of *API-5* to inhibit reporter activity because no inhibition of reporter activity was observed when the miR-224-binding sites in *API-5* 3′-UTR was mutated (Fig. 3*D*). We further demonstrated that miR-224 can inhibit endogenous *API-5* expression (Fig. 3*E*), which is consistent with our earlier observation that increased miR-224 expression leads to increased cell death (Fig. 2*D*) because *API-5* is an anti-apoptotic gene.

Earlier studies suggest that miRNA acts at the translational level because only significant differences in the protein but not the mRNA levels were observed (51). Our observations that increased miR-224 reduced the endogenous *API-5* mRNA levels in cells thus suggest that it is likely that miR-224 inhibit *API-5* expression at the post-transcriptional level by degrading *API-5* transcript, resulting in reduced steady-state *API-5* transcript levels (Fig. 3*E*). Reduced *API-5* levels will then sensitize these cells to apoptosis (Fig. 2*D*).

To evaluate the functional consequences of inhibiting the endogenous expression of miR-224 in primary liver cells, miR-224 inhibitor was transfected into THLE-3 cells, an immortalized primary liver cell line. Inhibiting miR-224 expression in these primary liver cells significantly inhibited ($p < 0.001$) the endogenous expression of miR-224 in these cells (Fig. 4*A*, *top panel*) and significantly increased the physiological expression of the *API-5* gene (Fig. 4*A*, *bottom panel*). Notably, reduced physiological levels of miR-224 expression in the THLE-3 primary liver cells was also found to protect these cells from UV-induced apoptotic cell death ($p < 0.05$) (Fig. 4*B*).

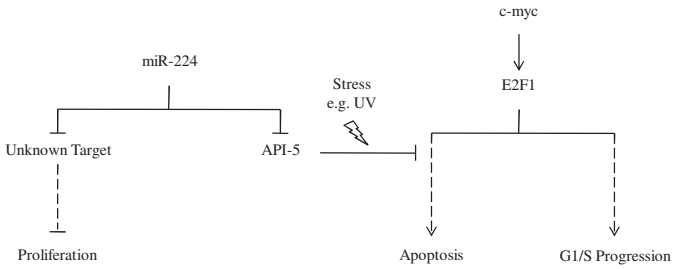


FIGURE 5. **Hypothetical model of the role of miR-224.**

Taken together, our data suggest that miR-224 influences both the proliferation and apoptotic potential of cells. It has been proposed that a single miRNA may regulate different unrelated target genes to control opposing activities like cell proliferation and apoptosis (52). miR-224 may thus represent such a miRNA. The regulation of the cell proliferation potential is likely to be mediated by a target gene of miR-224, which is currently unknown, whereas the regulation of the apoptotic potential is likely to be mediated via *API-5*, a known gene that regulates apoptosis (Fig. 5). *API-5* was previously reported to act downstream E2F and inhibit E2F-dependent apoptosis without affecting E2F-dependent transcription (42). We thus hypothesize that miR-224 may influence E2F-dependent apoptosis via *API-5*.

Notably, there is a significant inverse correlation between miR-224 expression and *API-5* expression in HCC patients ($p < 0.05$) (Fig. 6). The significant correlation between miR-224 and *API-5* in HCC patients lends credence to the experimental observation that miR-224 negatively regulates *API-5* expression and highlights the clinical relevance of this observation.

The observation that miR-224 is overexpressed in the tumors of HCC patients and that it plays a role in sensitizing cells to apoptosis via the inhibition of *API-5* expression seems to contradict conventional wisdom that apoptosis is reduced during carcinogenesis. Nonetheless, similar to miR-224, oncoproteins such as c-Myc and E1A were found to sensitize cells to apoptosis upon minor insults that normal cells can usually resist, for example, serum depletion, DNA-damaging agents, hypoxia, etc. (53, 54). Oncogenic changes that promote apoptosis are thought to provide the selective pressure for cells to override apoptosis during the multistage process of carcinogenesis (53), resulting in a resistant population of cells that accumulate heritable genetic mutations (55) during its increased lifespan, thus facilitating oncogenic transformation. The dual role of miR-224 to influence both cell proliferation and cell death simultaneously may thus potentially hasten this selection process favoring cells that accumulate sufficient heritable genetic mutations to override apoptosis during carcinogenesis.

In summary, we have identified 19 up-regulated and three down-regulated miRNAs that are associated with HCC. Pre-

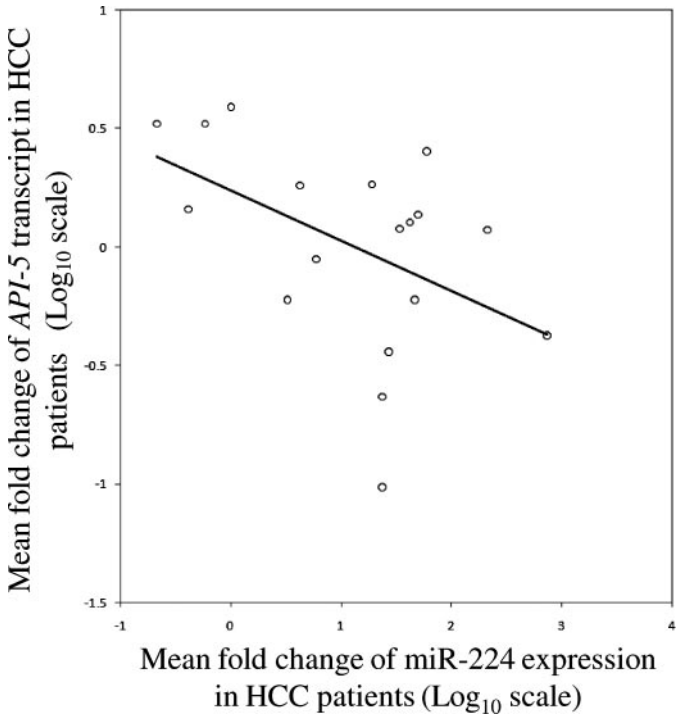


FIGURE 6. **API-5 transcript expression level is inversely correlated with miR-224 expression in HCC patients.** Scatter plot of the mean fold change of *API*-5 transcript *versus* that of miR-224 expression. Each spot represents data from one patient presented in $\log_{10}$ scale, and a linear regression line is depicted as a *solid line*. Statistically significant inverse correlation was observed between *API*-5 and miR-224 expression ($p < 0.05$ and $R^2 = 0221$).

dicted putative gene targets of these differentially expressed miRNAs in HCC were found to belong to 34 significant biological processes, most of which have been implicated in carcinogenesis. Further characterization of one of these dysregulated miRNAs revealed that miR-224, which is up-regulated in the tumors of HCC patients, sensitizes cells to apoptosis through the inhibition of *API*-5 at the mRNA levels and increases cell proliferation. Significantly, miR-224 expression was found to be inversely correlated with *API*-5 expression in HCC patients. These findings reaffirm the important role of miRNAs in regulating gene expression and suggest that dysregulation of the expression of miRNAs may lead to dysregulated target gene expression resulting in dysregulated cellular processes that may ultimately lead to tumorigenesis.

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MicroRNA and cancer – focus on apoptosis

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Abstract

MicroRNAs (miRs) are small non-coding RNAs regulating gene expression at the post-transcriptional and/or translational levels. miRs play important roles in diverse biological processes, including development, cell differentiation, proliferation and apoptosis. Recent evidence has shown that miR loci frequently map to cancer-associated genomic regions and deregulated miR expression profiles are associated with many cancer types, implicating miRs in crucial processes that lead to tumourigenesis. Here, we review the current findings about miRs and tumourigenesis, focusing on their involvement in the apoptosis pathway. A significant observation is that greater than one-quarter of all known human miRs were reported to be deregulated in at least one cancer type. The expression of a subset of miRs (*e.g.* miR-21 and miR-155) was found to be consistently up-regulated, whereas another subset of miRs (*e.g.* miR-143 and miR-145) was consistently down-regulated across different cancer types suggesting their involvement in regulating common cellular processes whose deregulation may lead to tumourigenesis. Several miRs were implicated to play roles in cell proliferation and apoptosis. Some miRs, such as miR-29b and miR-15-16, influence only the apoptotic pathway, whereas others including let-7/miR-98 and miR-17-92 may play roles in both the apoptotic and cell-proliferation pathways. In conclusion, although our current understanding of the functions of miRs is still fragmentary, taken together, this review highlights the complex and intricate roles that miRs play in the regulation of cellular processes. Perturbation of the expression of miRs may thus lead to tumourigenesis.

Keywords: microRNAs • tumourigenesis • apoptosis

Introduction

microRNAs (miRs) are a class of small non-coding RNAs whose mature products are ~22 nucleotides long. They negatively regulate gene expression at the post-transcriptional and/or translational level. They were first discovered by Ambros and colleagues in 1993 [1] in *C.elegans* and were shown to be abundantly expressed in viruses [2], plants [3] and animals [4]. To date, there are a total of 6396 miRs (miRBase Release 11. http://microrna.sanger.ac.uk/sequences/), of which, 678 miRs are found in human beings [5–7]. Many miRs show sequence and function conservation between distantly related organisms, suggesting that this class of small RNAs is an integral part of essential cellular processes [8]. For example, Lethal-7 (Let-7) was initially discovered to be responsible for the developmental transition of L4 lavae to the adult cell fates [9] in *C.elegans*. It was later found to be evolutionarily conserved, regulating development in *Drosophila*, zebrafish, annelids, mollusks [8] and mouse [10] and possibly

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human beings, which comprised 12 members of the Let-7 family. Their strong evolutionary conservation suggests that they are likely to have an ancient origin [11] although they were identified only recently. Their discovery has opened up a new dimension in our understanding of gene regulation.

miR biogenesis

microRNAs are encoded in the genome and transcribed by RNA polymerase II as primary transcripts that are called pri-miRs. Pri-miRs are typically 3 to 4 kilobases long single-stranded RNAs with 5′cap, 3′ poly(A) tail and complicated secondary structure [12, 13]. The Pri-miRs are processed in the nucleus into one or more precursor-miRs (pre-miRs) of ~70-nucleotide by microprocessor complex comprising the nuclear RNase III, Drosha, and the double-stranded RNA binding protein, Pasha/DGCR8 [13–15]. Pre-miRs are then actively exported to the cytoplasm through exportin-5 in association with RAN-GTPase [16, 17]. In the cytoplasm, another RNase III, known as Dicer, further processes the pre-miR into ~22-nucleotide mature miR, which is double-stranded (miR duplex). The miR duplex [18, 19] comprises a strand (miR strand), which is incorporated into the multi-protein RNA-induced silencing complex (miRISC) and a complementary strand (miR* strand), which is degraded. Thermodynamic stability of the strand probably determines the choice of strand to be incorporated into miRISC complex [20]. In mammalian system, the functional miRISC carrying the mature miR can bind to the 3′untranslated region (3′UTR) of its target gene mRNA to result in either mRNA degradation (for nearly perfect complementary base-paring) or protein translation inhibition (for imperfect complementary base-paring). The mechanism of inhibition will depend on the miR sequence, the target mRNA sequence and the exact composition of the miRISC protein complex [21, 22].

miR and cancer

The importance of microRNAs in cancer is highlighted by the observation that ~50% of miRNA genes are located in cancer-associated genomic regions or fragile sites [23, 24], which are frequently amplified or deleted in tumourigenesis. Global repression of microRNA processing machinery (Drosha, Pasha/DGCR8 and Dicer1) promotes cellular transformation and miRNA processing-impaired cells formed tumours with accelerated kinetics in mouse model, implicating the role of mature miRs in cancer-related processes [25]. Large-scale microRNA expression profiling of human cancers have revealed that miRNA deregulation is frequently associated with many cancer types including those originating from the blood [26–31], brain [32–34], thyroid [35–37], breast [38], lung [39–41], tongue [42], nose and pharynx [43], liver [44–47], the gastro-intestinal system (esophageal [48], gastric [49], pancreatic [50, 51] and colorectal cancers [52, 53]) as well as the genitourinary system (cervical [54], ovarian [55, 56] and prostate [57, 58] cancers).

Table 1 summarizes our current knowledge on the profile of miR expression in various human cancers. In these studies, miR expression in tumours is compared against paired non-tumourous tissues from cancer patients and significantly up- and down-regulated miRs are indicated with red-box/up-arrow and green-box/down-arrow, respectively. More than one-quarter of known human miRs (175 out of 678 miRs) have been reported to be significantly deregulated in at least one cancer type. However, this may be a gross underestimation of the actual numbers of deregulated miRs as the majority of the known miRs were only identified in the previous 2 years and were not included in earlier miR expression profiling studies. Nonetheless, this observation suggests that microRNAs may represent one of the largest classes of gene regulators implicated in cancer-related processes although very little are known about them. Table 1 also highlights some interesting patterns of miR expression profiles in cancers. Of the cancer-implicated miRs, miR-21 is the most commonly up-regulated miR in both solid and haematological tumours, consistent with the report of Volinia *et al.* [58]. Besides miR-21, other miRs including miR-155, miR-181b, miR-221 and miR-222 are also frequently up-regulated in cancers of the blood, brain, thyroid and the gastro-intestinal (GI) systems, and to a lesser extent in liver cancer, lung cancer and breast cancer. In contrast, the let-7/miR-98 cluster is commonly down-regulated in tumours of the thyroid, breast, lung, upper GI and the genitourinary system. Similarly, miR-143 and miR-145 are frequently down-regulated in the haematological tumours and solid tumours of the breast, lung, prostate and the lower GI system. Such common deregulation of miR expressions across various tumour types suggests that these miRs may be involved in crucial cellular pathways that are commonly deregulated in cancer development. Indeed, functional studies have demonstrated that let-7/miR-98 negatively regulate RAS [59] and v-myc myelocytomatosis viral oncogene homologue (MYC) [60] whilst miR-21 negatively regulate phosphatase and tensin homologue (PTEN) [44] and programmed cell death 4 (PDCD4) [61], which are proto-oncogenes or tumour suppressors that regulate important cellular processes, including cell growth, proliferation and apoptosis whose deregulation leads to tumourigenesis. In addition to miRs that are commonly deregulated across different cancers, there are also miRs that seem to be deregulated in only specific tumour types (Table 1). For example, the miR-17-92 cluster and miR-93 are frequently reported to be up-regulated mainly in cancers of the GI system. Interestingly, a very large proportion of miRs (~81%) were found to be up-regulated in thyroid tumours, whereas a high percentage of miRs (~70%) were reported to be down-regulated in prostate cancers. These observations suggest that some tumour-specific mechanisms may be in place to favour particular miR profiles depending on the tumour micro-environment. It is also worth noting that miR-105, miR-144, miR-193 and miR-199b are seldom reported to be deregulated in cancer, despite their relatively early discovery, suggesting that these miRs probably play a role in cellular

Table 1: miRs that are significantly differentially expressed in human cancers and their validated *in vivo* targets.

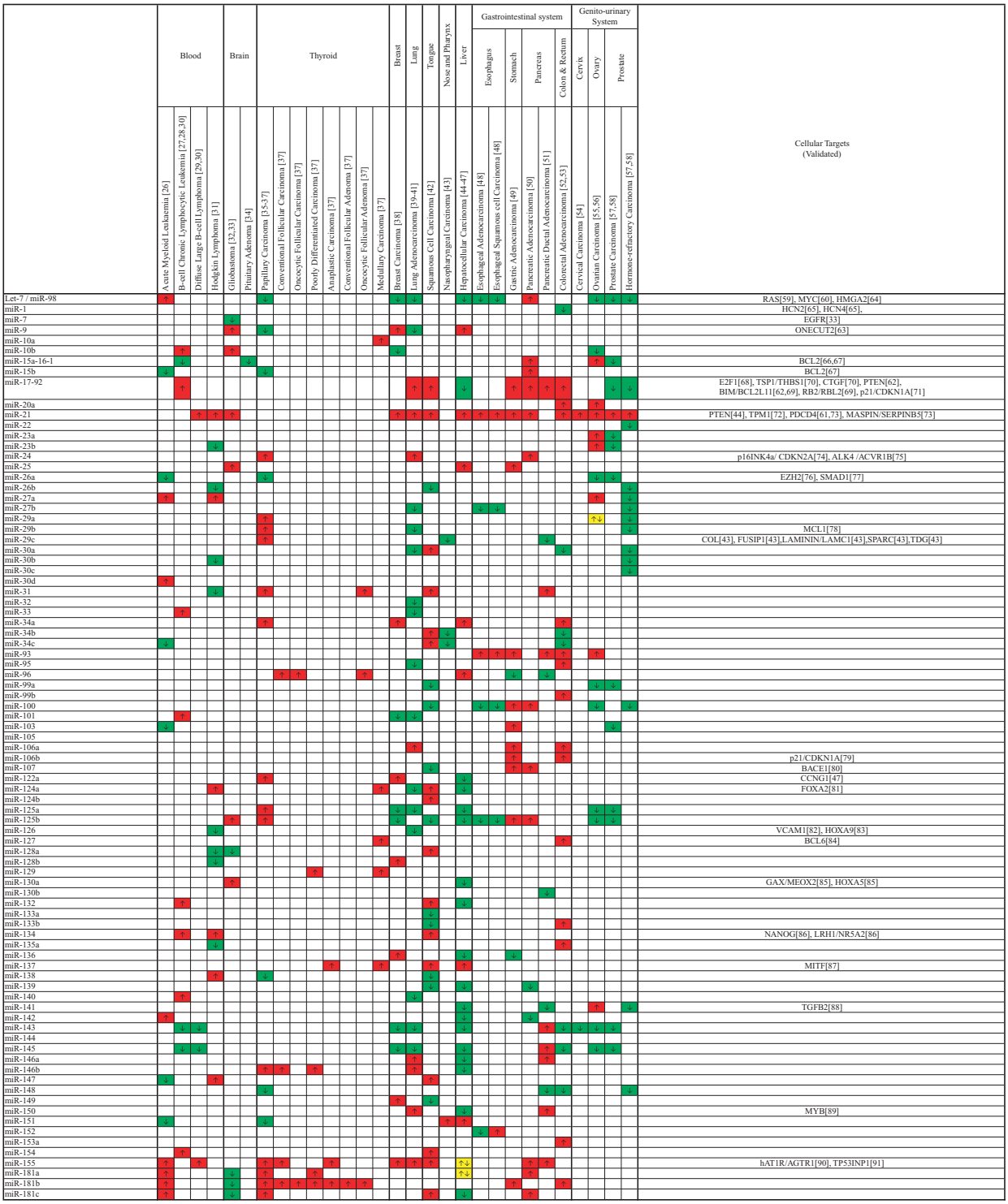

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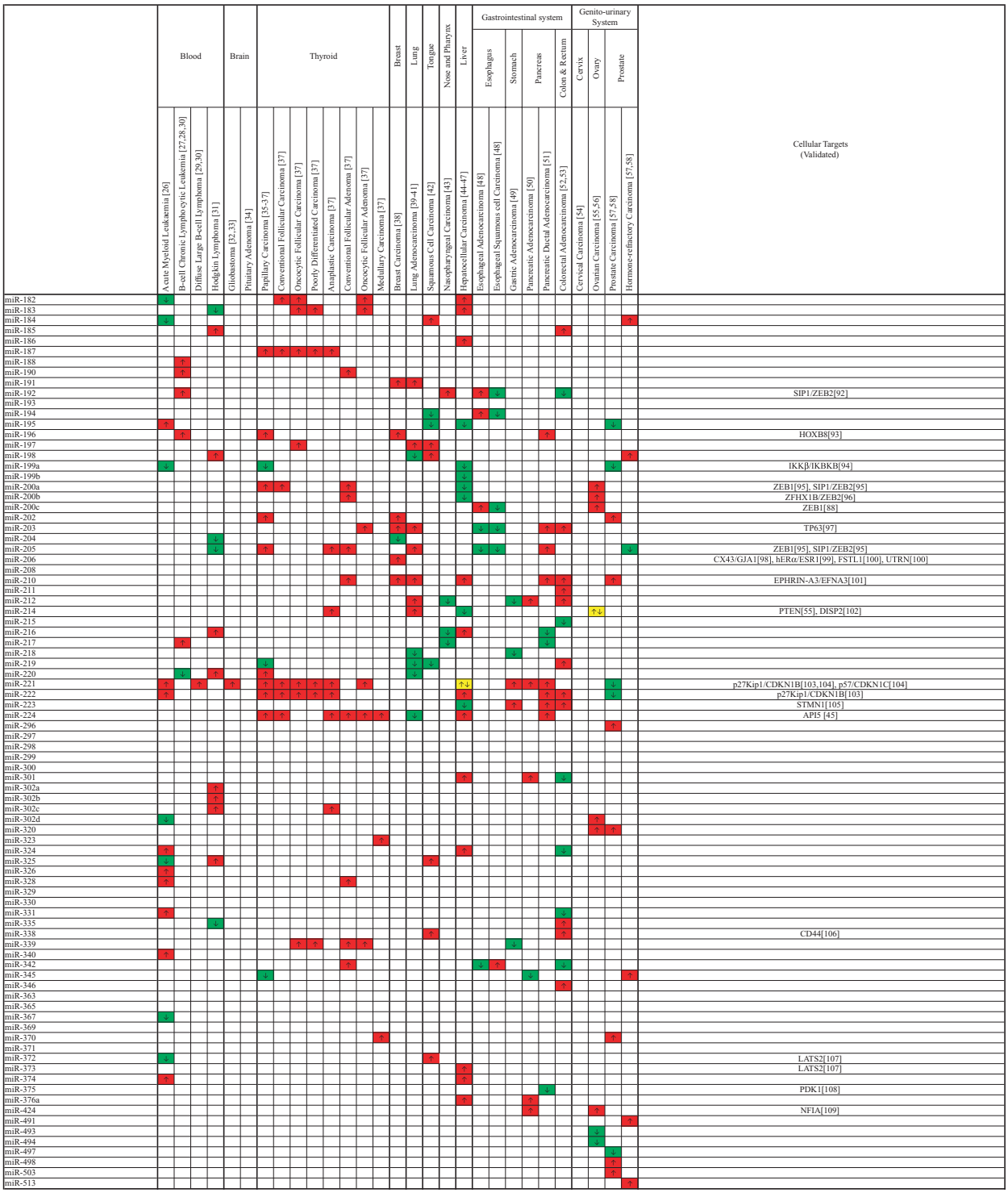
Table 1: Continued



Upward pointing arrows in red boxes indicate that the miR is up-regulated in the cancer-type while downward pointing arrows in green boxes indicate down-regulated miRs. Yellow boxes with both Upward- &downward-pointing arrows indicate that miR was reported to be up-regulated in one study but down-regulated in another study.

house-keeping processes and are less likely to be involved in oncogenesis.

Although much is known about the aberrant miR expression pattern associated with various cancers, much less is known about the functional relevance of such miR deregulation or the *in vivo* miR targets. Table 1 also summarizes a total of 65 non-overlapping experimentally validated direct cellular targets of miRs that are reported to date [33, 43–45, 47, 55, 59–109]. Table S1 annotates these validated targets based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. These 65 validated miR target genes show a significant enrichment in the classical cancer-associated pathways such as transcription, cell–cell adhesion and signalling, cell-cycle regulation, cell proliferation and apoptosis, strongly suggesting that the deregulation of these miR target genes may play significant roles in carcinogenesis. However, as predicted by miR target prediction algorithms (miRanda [6], PicTar [110] and TargetScan [111]), each individual miR can potentially regulate hundreds of cellular gene targets. But reports of the identification and characterization of these *in vivo* miR targets remain few, which prevent our comprehensive understanding of the miR-regulated networks that significantly impact cell differentiation, cell proliferation and apoptosis [112]. Current knowledge on a limited number of miRs or miR clusters has revealed the complexity of miR-regulatory networks, and in this review, we will discuss the role of a few well-studied miRs in tumourigenesis with a focus on its impact on the apoptotic pathway.

miR and apoptosis

Apoptosis is the intrinsic cellular mechanism to eliminate cells that are damaged or transformed. Deregulation of apoptosis is an important step in cancer as it allows the genetically unstable cells to survive and accumulate further mutations that eventually lead to tumourigenesis. As cancer cells are mostly characterized by increased cell proliferation and decreased cell death, cancer-implicated genes have conventionally been classified into two groups. One group, the oncogenes, up-regulates proliferation and down-regulates apoptosis, whereas the other group, the tumour suppressor genes, performs just the opposite function. Indeed, pro-apoptotic genes such as p53 are frequently inactivated whilst anti-apoptotic genes such as B-cell CLL/lymphoma 2 (BCL2) are frequently over-activated in cancer progression. However, recent evidence has shown that up-regulation of MYC and E2F oncogenes can increase both cell proliferation and apoptosis [113, 114], suggesting the classification of cancer-related genes into oncogenes or tumour suppressors may be an over-simplification. Figure 1 summarizes our current knowledge of miRs implicated in cell-proliferation and apoptosis, revealing that the miR-regulatory network is just as complicated as its protein-coding counterparts. Some miRs, such as miR-29b and miR-15-16, were found to affect only the apoptotic pathway, whereas others including let-7/miR-98 and miR-17-92 play roles in both the apoptotic and cell-proliferation pathways (see review [114, 115]). In the following discussion, we will discuss some pro-apoptotic miRs, anti-apoptotic miRs and miRs that regulate both proliferation and apoptosis.

The pro-apoptotic miRs targeting the BCL2 family of genes

The miR-15-16 cluster induces apoptosis by targeting the important anti-apoptotic factor BCL2 at the post-transcriptional level [66]. It was proposed to function as a tumour suppressor by keeping cell growth in check under normal physiological conditions. Like many tumour suppressors, this miR cluster is found to be frequently deleted in B-cell chronic lymphocytic leukaemia (CLL), resulting in its down-regulation in more than 68% of the CLL cases [28]. The miR-15-16 cluter is also reported to be down-regulated in pituitary adenoma [34] and prostate carcinoma [57]. Hence in these cancers, miR-15-16 expression is preferentially down-regulated to favour cancer development by inhibiting apoptosis (Fig.1). In a recent study, which utilized expression microarray to investigate the effects of miR-15a and miR-16-1 on the transcriptome and proteome of MEG-01 leukaemic cells, genes (*e.g.* MCL1, ETS1 and JUN) that directly or indirectly play a role in apoptosis and cell-cycle were found to be significantly differentially expressed in these cells. Another miR, miR-29b, which is down-regulated in lung and prostate cancers (Table 1), was reported to also target myeloid cell leukaemia sequence 1 (MCL1) [78], a member of the BCL2 family, implying that the function of miR-29b may be similar to that of miR-15-16. Curiously, in some cancers, the expression of these miRs (miR-15-16 and miR-29b) was reported to be up-regulated instead. One possible explanation to these seemingly contradictory observations may perhaps be that these miRs may deregulate other cellular processes in addition to apoptosis in these specific cancers.

The anti-apoptotic miR-21 targets PTEN and PDCD4

miR-21 is the most consistently up-regulated miR across many cancer types (Table 1). miR-21 was first implicated as an anti-apoptotic factor by the observation that knock-down of miR-21 increased apoptotic cell death in human glioblastoma cells [116] and in the mouse model [117]. miR-21 directly targets PTEN whose down-regulation will release its inhibition on protein kinase B (PKB) resulting in significantly reduced apoptosis in cancer cells (Fig.1). miR-21 also targets PDCD4 [61], a pro-apoptotic gene frequently down-regulated in hepatocellular carcinoma(HCC) [118]. Interestingly, miR-21 was also reported to be up-regulated in HCC (Table 1). This suggests that miR-21 can inhibit apoptosis through both PTEN and PDCD4. Recently, miR-21

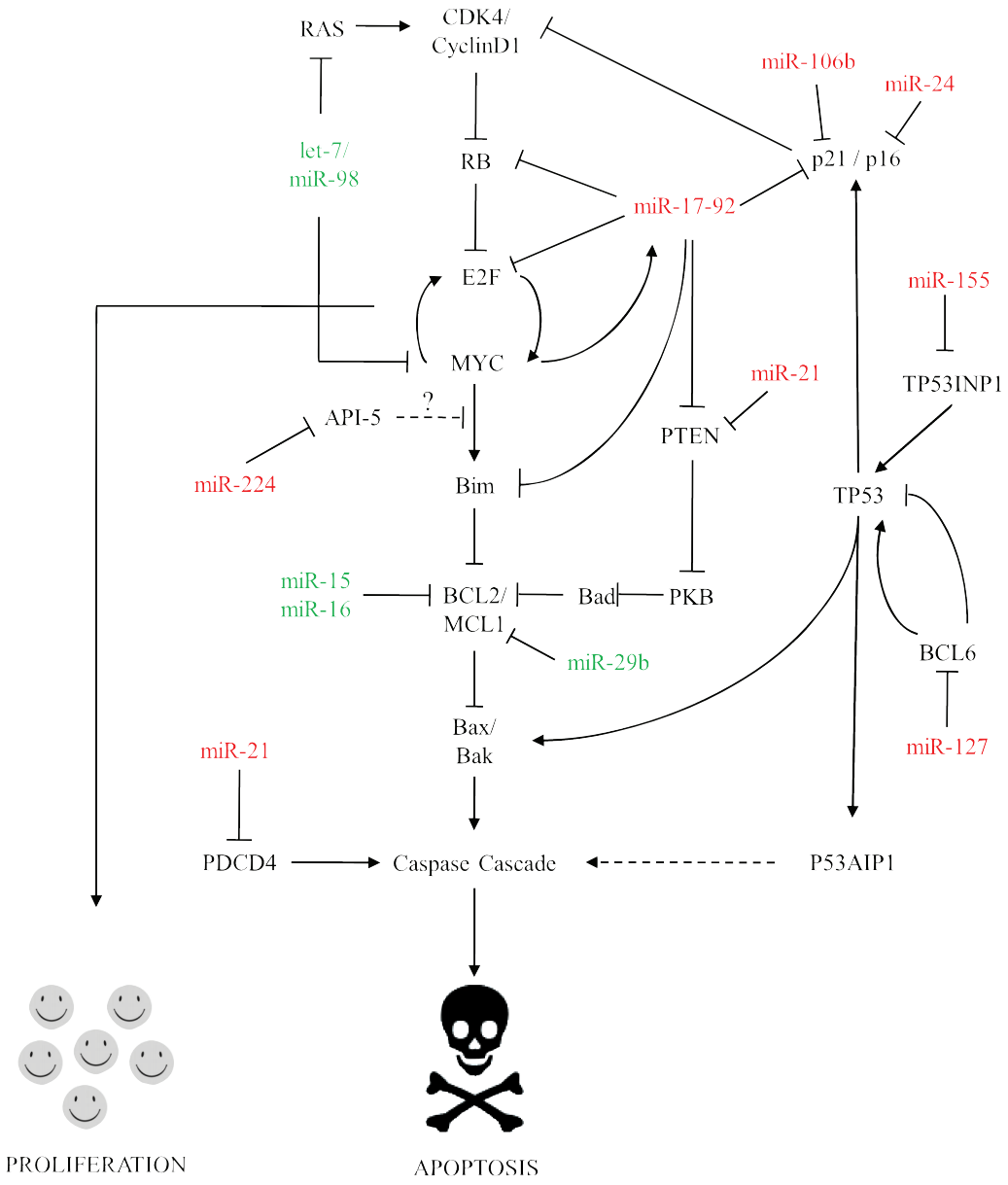


Fig. 1 Diagrammatic representation of the roles of miRs in the regulation of cell proliferation and apoptosis. Red colour indicates a general up-regulation of miRs in cancers and green colour indicates a general down-regulation of miRs in cancer.

was reported to target important tumour suppressor genes including tropomyosin 1 (TPM1) [72] and serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5) [73] suggesting that miR-21 may also play a role in tumour invasion and metastasis. Hence, the oncogenic potential of miR-21 lies in its ability to regulate multiple cancer-associated pathways probably *via* multiple cellular targets, which may partially explain its frequent up-regulation in cancer.

miR-210 decreases proapoptotic signalling in a hypoxic environment

Hypoxia-regulated microRNAs such as miR-210 is induced in response to low oxygen and play a role in cell survival by decreasing caspase activation, the central components of apoptotic signalling [119]. As hypoxia is an important feature of tumour microenvironment, it is of interest to note that miR-210 is also

over-expressed in many major tumour types (Table 1), suggesting that hypoxia may represent a contributing factor for microRNA deregulation in certain cancers. A recent study by Camps *et al.* has demonstrated that miR-210 is a good prognostic marker for breast cancer [120].

Let-7/ miR-98 family and possible co-operation with miR-21

Our understanding of the role of let-7/miR-98 family in cancer development was facilitated by the identification of two proto-oncogenes regulating cell proliferation and apoptosis, RAS [59] and MYC [60], as direct targets of let-7/miR-98. Under normal physiological conditions, Let-7 regulates cellular proliferation by inhibiting RAS and MYC expression. However, in tumours, let-7/miR-98 are frequently down-regulated resulting in increased expression of cellular RAS and MYC and subsequent elevation of cell proliferation as well as MYC-induced apoptosis [114] (Fig.1). Increased apoptosis and proliferation seem to contradict the conventional wisdom that apoptosis is reduced during carcinogenesis. However, oncogenic changes that promote apoptosis are thought to provide the selective pressure for the cells to override apoptosis during the multistage process of carcinogenesis [121], resulting in the final cell population that retain high proliferative but reduced apoptotic potential. It is important to note that expression of miR-21 is frequently up-regulated in let-7/miR-98 down-regulated tumours of the thyroid, breast, lung, liver, esophagus and prostate (Table 1). This suggests that miR-21 or other cellular factors may counter-balance MYC-induced apoptosis in tumours in which let-7/miR-98 expression is down-regulated, whereas still maintaining a high rate of cell proliferation.

miR-17-92 cluster highlights the complexity of miR regulatory networks

The miR-17-92 cluster, which modulates E2F1 expression, is positively regulated by MYC [122]. Under normal physiological conditions, miR-17-92 facilitates the tight regulation of MYC-mediated cellular proliferation by inhibiting the MYC-induced E2F1 expression. However, when miR-17-92 is over-expressed as in the case of GI cancers, it can potentially become a very potent oncogene targeting multiple cellular pathways to favour tumourigenesis by enhancing cell proliferation and inhibiting apoptosis. As illustrated in Fig.1, miR-17-92 can increase MYC-enhanced proliferation by targeting p21 [71] and consequently activating the CyclinD1/CDK4 complex to release retinoblastoma (RB)'s inhibition on E2F. In addition, miR-17-92 is also capable of down-regulating RB [69] directly to drive cell proliferation. On the other hand, miR-17-92 is also capable of minimizing MYC-induced apoptosis by targeting BCL2-like 11 (BIM) and PTEN [62] to increase the level of anti-apoptotic BCL2. Hence, miR-17-92 is truly worthy of its reputation as the first non-coding oncogene, oncomiR-1 [123]. This miR demonstrates the complexity of miR regulatory network.

miR-224, the double-edged sword

miR-224 is up-regulated in HCC, pancreatic ductal adenoma and various types of thyroid cancers (Table 1). We have demonstrated that over-expression of miR-224 sensitizes cells to apoptosis through API-5, an apoptosis inhibitor, and increase cell proliferation through yet an unknown mechanism [45]. Sassen *et al.* has previously proposed that a single miR can potentially regulate opposing cellular activities such as cell proliferation and apoptosis [124] and miR-224 represents the first such miR identified. Similar to the MYC oncogene, which regulates both cell-proliferation and apoptosis, the dual role of miR-224 to influence both cell proliferation and apoptosis can potentially hasten the selective process favouring cells that accumulate sufficient heritable genetic mutations to override apoptosis during the multistage of carcinogenesis.

Other miRs implicated in apoptosis

There are a number of other miRs that may potentially play roles in regulating apoptosis in cancer. For example, miR-155 is frequently over-expressed in many cancers and targets the tumour protein p53 inducible nuclear protein 1 (TP53INP1) [91]. TP53INP1 was reported to be a positive regulator of p53-dependent apoptosis by enhancing Ser46 phosphorylation of p53 which in turn induced p53-regulated apoptosis-inducing protein 1(p53AIP1) expression and subsequent apoptotic cell death [125]. Hence, over-expression of miR-155 in cancers will inhibit TP53INP1 expression and attenuate apoptotic cell death induced by TP53INP1. In contrast, miR-127 was reported to target B-cell CLL/lymphoma 6 (BCL6) [84] to potentially increase TP53-dependent apoptosis by disrupting the negative regulatory feedback loop between BCL6 and TP53 [126, 127]. However, our understanding of the rationale behind this deregulation in cancer remains unclear.

Conclusion

One of the hallmarks of cancer is defects in the regulatory circuits that control normal cell proliferation and homeostasis. Previously, great efforts were focused on understanding the roles of protein-coding genes in cancer. As discussed above, emerging research are implicating miRNAs as a novel class of non-coding tumour suppressors and oncogenes that play important roles in tumourigenesis. As we review the roles of miRNAs in apoptosis and

cancer, we begin to appreciate that miR's role in tumourigenesis is not merely either pro- or anti-apoptosis. Rather, it is likely that coordination and perhaps synergism between several deregulated miRs and their protein-coding counterparts facilitate a favourable environment for cancer formation. Although current knowledge of miR function and targets is incomplete, it underscores the complexity of the roles of RNA in the regulation of cellular pathways. Continued effort in the detailed characterization of miR target and function is necessary to improve our understanding of the role of miRs in tumourigenesis and facilitates the design of appropriate therapies targeting this novel group of molecules.

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Lethal-7 is down-regulated by the hepatitis B virus x protein and targets signal transducer and activator of transcription 3

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Background & Aims: The pleiotropic hepatitis B virus (HBV) x protein (HBx), associated with hepatocellular carcinoma (HCC), has been implicated in the deregulation of cellular gene expression at the transcriptional level. To date, it remains unknown if HBx regulates the expression of miRNAs which play important roles in gene-regulation at the post-transcriptional and/or translational level.

Methods: miRNA microarrays were employed to compare the expression of cellular miRNAs in HBx-versus control-HepG2 cells. Reverse-transcription Taqman realtime-PCR was used to examine let-7a expression in normal liver as well as paired HCC-tumor and adjacent non-tumorous liver. Let-7a miRNA was functionally characterized in cells with transiently altered let-7a expression. The direct target of let-7a was identified *in silico* and validated using 3′UTR-reporter assay.

Results: HBx up-regulates 7 and down-regulates 11 miRNAs, including the let-7 family. HBx expression was found to have a significant inverse correlation with the expression of the highly-expressed members of the let-7 family in HCC patients, highlighting the clinical relevance of our observations. Further characterization of let-7a, the most highly expressed let-7 family member, revealed that it negatively regulates cellular proliferation partly through targeting signal transducer and activator of transcription 3 (STAT3). HBx-mediated down-regulation of let-7a and up-regulation of STAT3 supports cell proliferation in HBx cells.

Conclusion: This study thus represents the first demonstration of HBx's ability to deregulate cellular miRNA expression. The deregulation of the expression of the let-7 family of miRNAs by HBx may represent a potential novel pathway through which HBx acts to deregulate cell proliferation leading to hepatocarcinogenesis.

Keywords: Hepatocellular carcinoma; Hepatitis B virus x protein; microRNAs; let-7; Signal transducer and activator of transcription 3.

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBx, hepatitis B virus x protein; miRNA, microRNA; 3′UTR, 3′ untranslated region; let-7, lethal-7; DMEM, Dulbecco's modified Eagle's media; Cy-3, cyanine-3; Cy-5, cyanine-5; PCR, polymerase chain reaction; CT, threshold cycle; B-gal, beta-galactosidase; FACS, fluorescence activated cell sorting; STAT3, signal transducer and activator of transcription 3.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer related death worldwide, accounting for ~700,000 deaths annually [1]. As much as 80% of cases of HCC, mostly occurring in countries where hepatitis B virus (HBV) is endemic such as South-East Asia and Sub-Saharan Africa, are attributed to chronic HBV infections [2]. It has been suggested that HBV promotes carcinogenesis through at least two major mechanisms: (1) Through the integration of viral genomic fragments into the human genome that can result in cis activation of proto-oncogenes or cis-repression of tumor suppressor genes. (2) Additionally, viral proteins can lead to trans-activation or trans-repression of cellular genes/pathways that may eventually lead to oncogenic transformation [2].

Hepatitis B virus x protein (HBx) is a ~17 kDa protein that is conserved among the mammalian hepadnaviruses and is implicated to play an important role in hepatocarcinogenesis. The HBx gene is the most common viral open reading frame integrated into the host genome of HCC patients, and the HBx protein is often found to be selectively over-expressed in HCC [3–5]. HBx lacks a DNA binding domain and is widely believed to function as a promiscuous trans-co-activator by interacting with various cellular factors to regulate, at the level of transcription, a number of cellular pathways such as JAK/STAT pathway [2,6], and many host genes such as p53 tumor suppressor (TP53). The exact role of HBx in HCC oncogenesis is not well understood and HBx protein has been shown to display pleiotropic properties by impacting on multiple, sometimes opposing pathways such as cell death and cell proliferation. HBx has been shown *in vitro* to transform immortalized murine hepatocyte cell lines [7–10], murine fibro-

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blasts [8], and rat fibroblast cell lines [11]. In addition, Kim et al. and Yu et al. have separately demonstrated that HBx can directly cause HCC in mouse models [12,13]. On the contrary, several other groups have found that HBx enhances cellular apoptosis *in vitro* [14–18] while Lee et al. reported that HBx is not tumorigenic in his line of transgenic mice [19]. Taken together, these reports suggest that HBx has the potential to regulate many cellular processes with seemingly opposite functions, and the predominant phenotype observed is dependent on other cellular factors and the microenvironment in which the function of HBx is evaluated. Although the role of HBx, in regulating gene expression through its interaction with transcription factors like TP53 [18], has long been established, it remains unknown if HBx regulates gene expression through the regulation of microRNAs (miRNAs), a class of small non-coding RNAs.

Thus far, ~700 miRNAs have been identified in the human genome (miRBase Release 12.0). They function by binding to the 3′ untranslated region (3′UTR) of their cellular targets to cause mRNA degradation and/or protein translation inhibition, both events resulting in a net reduction of functional target protein in the cell [20]. The miRNAs represent another important level through which gene expression is regulated, hence they play central roles in diverse cellular and developmental processes, and deregulation of miRNA expression may lead to cancer development [21]. Large-scale miRNA expression profilings of human cancers have revealed that miRNA deregulation is frequently associated with many solid and liquid tumors including human HCC [22–26], highlighting the role of miRNA in cellular processes involved in malignant transformation. An example of a miRNA implicated in carcinogenesis is the miRNA lethal-7 (let-7) which represents the first miRNA identified in humans. Let-7 was found to be frequently down-regulated in many human cancers such as tumors of thyroid, breast, lung, esophagus, ovary, prostate, and liver [27].

Since each miRNA can potentially regulate the expression of numerous gene targets in the cell, HBx's potential effect on cellular miRNAs may be a novel mechanism through which HBx deregulates cellular gene expression, and may partially explain the pleiotropic nature of this HBV viral protein. To investigate whether the deregulation of gene expression by HBx also occurs through the deregulation of cellular miRNA expression, we characterized the expression profiles of 286 miRNAs in HBx-expressing compared to control-HepG2 cells using miRNA microarrays. We identified a set of significantly differentially expressed miRNAs that is regulated by HBx, including members of the let-7 family. We demonstrated the inverse correlation between the expression of HBx and that of the highly-expressed members of the let-7 family including let-7a, let-7b, and let-7c in HCC patients, highlighting the clinical relevance of our observations. We further functionally characterized let-7a and identified its gene target.

Materials and methods

Cell lines and HCC patient samples

The human hepatocellular carcinoma HepG2 and SNU-182 cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal calf serum (Bioindustries, Isreal) and 25 mM HEPES (Invitrogen, USA). The human colorectal carcinoma HCT116 cells were cultured in modified McCoy's 5A media supplemented with 10% fetal calf serum (Bioindustries, Isreal). The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Paired tumorous and adjacent non-tumorous liver tissues from 20 hepatocellular carcinoma patients were obtained from the National Cancer Centre of Singapore Tissue Repository with prior approval from the National Cancer Centre (NCC) Institutional Review Board (IRB) (NCC IRB No.: NC08–12). As it is ethically not possible to obtain normal livers from healthy individuals, the 'normal' non-tumorous portion of the livers from 20 colorectal cancer patients with liver metastasis was obtained when these patients underwent surgery to remove the colorectal metastatic tumor in the liver. These tissues were also obtained from the National Cancer Centre of Singapore Tissue Repository with prior approval from the National Cancer Centre (NCC) Institutional Review Board (IRB) (NCC IRB No: NC05–022). The non-tumorous livers from colorectal cancer patients represent 'normal' liver in this study.

miRNA microarray

HepG2 cells were transduced with either HBx-expressing (HBx) or control (Control) recombinant adenovirus as previously described [18]. Cells were harvested 72 h post transduction for microarray analysis. Ambion mirVana™ Bioarrays were used to examine the miRNA expression. Significantly differentially expressed miRNAs were identified with Volcano Plot analysis.

RT-qPCR and Western blot analysis

Let-7 expression was measured with Taqman microRNA assay. STAT3 transcript expression was measured with SYBR RT-qPCR, and protein expression was measured with Western blot with specific antibodies.

Apoptosis and proliferation

Annexin V apoptosis assay and WST-1 cell proliferation assay were used to functionally characterize cells with transiently altered let-7a expression.

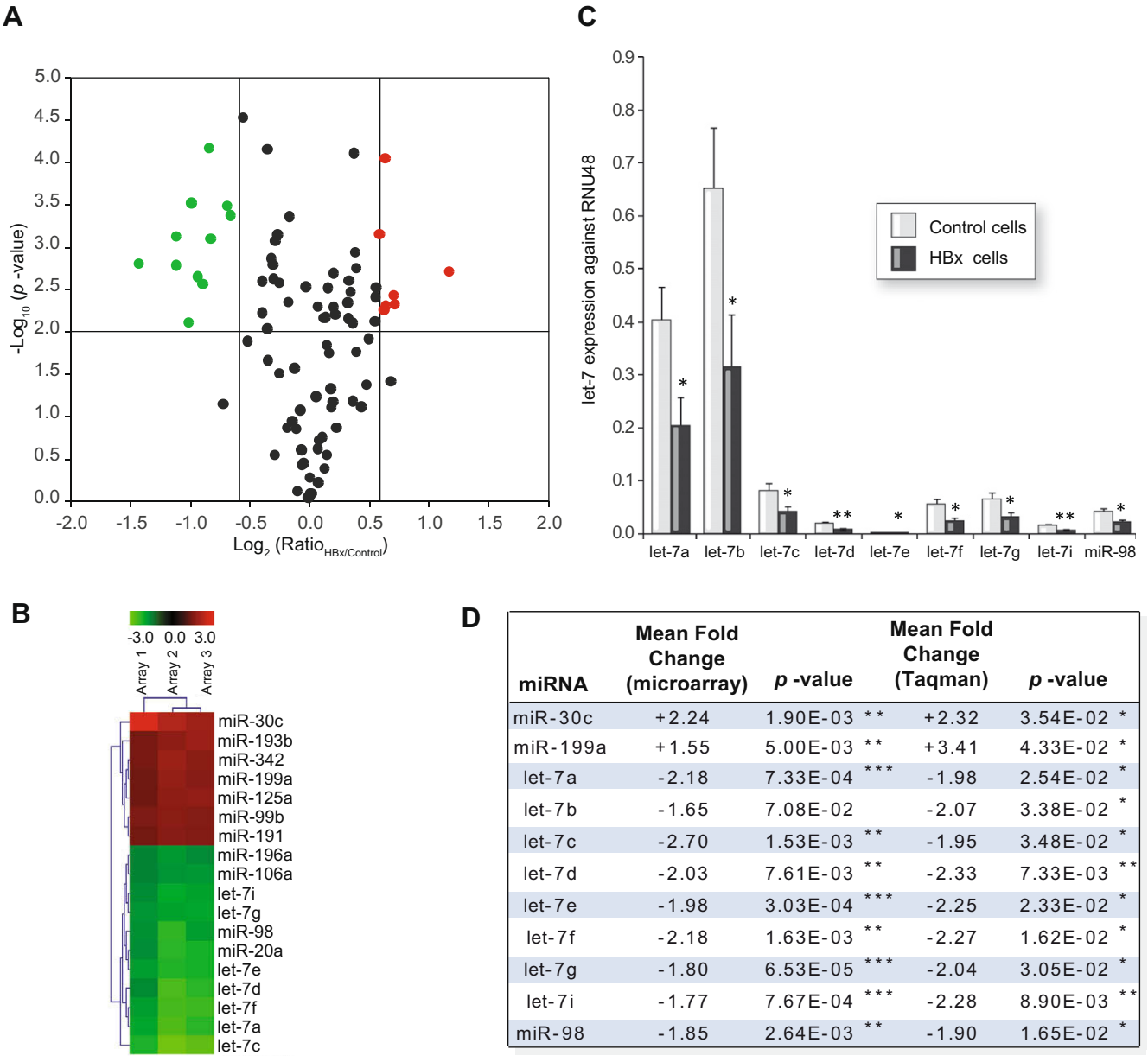
Detailed description of the materials and methods used can be found in the supplementary file.

Results

Let-7 family of miRNAs is significantly down-regulated in HBx-expressing HepG2 cells

To evaluate if HBx deregulates the expression of cellular miRNAs, the profile of 286 human miRNAs was examined in HBx-expressing versus control-HepG2 cells, in three independent experiments. The miRNA microarray data can be obtained from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=tlmdlkmyimcewhm&acc=GSE17474, Accession number: GSE17474.

Ninety miRNAs were found to exhibit significant expression in all three paired biological samples. These miRNAs are presented in Fig. 1A in a Volcano plot which displays the logarithmic transformation to the base 2 of the mean fold change of miRNA expression in HBx versus control cells, against the negative logarithm to the base 10 of the *p*-value of Student's *t* test. Significantly differentially expressed miRNAs were identified as absolute mean fold change of greater than 1.5 and *p*-value of less than 0.01, and located in the two upper-lateral quadrants. Seven miRNAs (miR-30c, miR-193b, miR-342, miR-199a, miR-125a, miR-99b, and miR-191) were found to be significantly up-regulated while 11 miRNAs (miR-196a, miR-106a, miR-20a, and let-7 family of miRNAs) were found to be significantly down-regulated in HBx-expressing cells compared to the control cells (Fig. 1B). Notably, we observed that eight out of the nine mature sequences of the human let-7 family were significantly down-regulated with the exception of let-7b which was also down-regulated, but with a *p*-value of 0.07 (Fig. 1D). Fig. 1B shows the 18 significantly differentially expressed miRNAs in a



D

miRNA	Mean Fold Change (microarray)	*p* -value		Mean Fold Change (Taqman)	*p* -value	
miR-30c	+2.24	1.90E-03	**	+2.32	3.54E-02	*
miR-199a	+1.55	5.00E-03	**	+3.41	4.33E-02	*
let-7a	-2.18	7.33E-04	***	-1.98	2.54E-02	*
let-7b	-1.65	7.08E-02		-2.07	3.38E-02	*
let-7c	-2.70	1.53E-03	**	-1.95	3.48E-02	*
let-7d	-2.03	7.61E-03	**	-2.33	7.33E-03	**
let-7e	-1.98	3.03E-04	***	-2.25	2.33E-02	*
let-7f	-2.18	1.63E-03	**	-2.27	1.62E-02	*
let-7g	-1.80	6.53E-05	***	-2.04	3.05E-02	*
let-7i	-1.77	7.67E-04	***	-2.28	8.90E-03	**
miR-98	-1.85	2.64E-03	**	-1.90	1.65E-02	*

Fig. 1. Profile of miRNAs deregulated by HBx in HepG2 cells. (A) Volcano plot showing 90 miRNAs that are significantly expressed in HBx cells. miRNA microarray data were collected by comparing the miRNA expression in HBx-expressing HepG2 cells (HBx cells) versus the control cells from three biological replicates. *Y*-Axis: $-\log_{10}(p\text{-value})$; *X*-axis: $\log_2(\text{ratio(HBx/control)})$. Differential expression of miRNAs in HBx cells versus control cells that are both biologically and statistically significant are indicated in the two upper-lateral quadrant with absolute fold change >1.5 and *p*-value <0.01. Green spots indicate miRNAs that are significantly down-regulated while red spots indicate miRNAs that are significantly over-expressed in HBx cells. (B) Hierarchical clustering of miRNAs that are differentially expressed in HBx-expressing cells using Euclidean correlation with average linkage. Up-regulated miRNAs are represented by red blocks while down-regulated miRNAs are shaded green. (C) Let-7 expression normalized against RNU48, measured using reverse-transcription Taqman real-time PCR. Data are presented as mean ± SE from three independent experiments. (D) Table showing the mean fold change and *p*-value of up-regulated miR-30c, miR-199a, and down-regulated let-7 miRNAs in HBx cells versus control cells from both the miRNA microarray and RT Taqman real-time PCR. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001.

Heat Map using unsupervised hierarchical clustering with Euclidean distance and average linkage analysis. We chose to validate the expression of two up-regulated miRNAs (miR-30c and miR-199a) and all the members of the let-7 family, using reverse-transcription Taqman real-time PCR. The data were normalized against the endogenous control RNU48. The let-7 family of miRNAs showed greatly variable endogenous level of expression. The expression of let-7a, let-7b, and let-7c was high whereas the expression of let-7e and let-7i was very low (Fig. 1C). Nonetheless, all 9 mature miRNAs were validated to be significantly down-regulated in HBx-expressing cells. (Fig. 1C and D). To ensure that the observed effect of HBx on let-7 is not cell line specific, we repeated the experiment in a second liver cell line, SNU-182 [28]. As evident in Supplementary Fig. S2, the expression of let-7a, let-7b, let-7c, let-7e, and let-7i was significantly reduced in HBx-expressing SNU-182 cells. Taken together, these results

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show a distinct miRNA expression profile, including the down-regulation of the entire let-7 family of miRNAs, which is associated with HBx in HepG2 cells. Moreover, the down-regulation of selected members of the let-7 miRNAs by HBx is reproducible in SNU-182 cells.

HBx expression is inversely correlated with let-7a expression in hepatocellular carcinoma patients

We proceeded to evaluate the clinical relevance of the observation that HBx negatively regulates members of the let-7 family, by examining the correlation between the HBx protein expression and the various let-7 family members in 20 HBV-associated HCC patients. Supplementary Fig. S3 shows the HBx status in the 20 HBV^+ HCC patients, analyzed through Western blot analysis with polyclonal rabbit-anti-HBx antibody generated from our laboratory. The expression of miR-98 was undetectable in the patients' samples. With the exception of let-7e and let-7i, which are expressed at very low levels in the HepG2 liver cell line, the expression of the other members of the let-7 family generally negatively correlates with the expression of the HBx protein in HCC patients (Supplementary Fig. S4), although the correlation is only statistically significant (p <0.05) in the three most highly expressed let-7 miRNAs (let-7a, let-7b, and let-7c) (Fig. 2A). Further examination of let-7a revealed that let-7a expression is significantly lower in tumors compared to the adjacent non-tumorous liver tissues of HCC patients (p <0.001), and both HCC-tumors and adjacent non-tumorous liver tissues show significantly lower let-7a expression compared to normal liver tissues (p <0.001 and p <0.01, respectively) (Fig. 2B). When the HBV^+ HCC patients were sub-grouped based on the relative HBx status between tumor and non-tumor tissues, let-7a expression was significantly lower while HBx expression was significantly higher in the tumor compared to the non-tumorous liver tissues in 16 HCC patients (p <0.001). In contrast, although not statistically significant, let-7a expression was higher while HBx expression was lower in the tumor compared to the non-tumorous tissues in the remaining four HCC patients (Fig. 2C). Hence, this significant negative correlation between the expression of let-7a and the expression of the HBx protein in HCC patients reaffirms the clinical relevance of our observations.

Let-7 negatively regulates cell proliferation

Let-7 miRNAs have been reported to play important roles in cell differentiation and cell proliferation (see review [29]). To evaluate the functional significance of HBx-mediated let-7 down-regulation, the level of cellular let-7 expression was altered by exogenously introducing the let-7 precursor or let-7 inhibitor into either HepG2 or SNU-182 cells. As the members of the let-7 family of miRNAs have previously been shown to have similar functions [30], let-7a was selected as a representative member of the let-7 family of miRNAs since it is one of the most highly expressed let-7 miRNA in our system, and consistently down-regulated by HBx in both HepG2 and SNU-182 cells (Fig. 1C, Supplementary Fig. S2). A significant 30% decrease (p <0.001) in cell proliferation (Fig. 3B, left panel) was observed when let-7a precursor was introduced into HepG2 cells (Fig. 3A, left panel). Inversely, when let-7a expression was inhibited by ~50% through the introduction of let-7a inhibitor (Fig. 3A, left panel), there was a corresponding significant increase (p <0.01) in cell proliferation (Fig. 3B, left panel) in HepG2 cells. The expression of unrelated miR-100 remained undisturbed, demonstrating the specificity of the small oligos in perturbing endogenous let-7a level. Similar results were observed in the SNU-182 cells (Fig. 3A, 3B, right panel). Our data confirm previous observations made by Johnson et al. [30] highlighting the role of let-7 in negatively regulating cell proliferation in HepG2 cells. No significant difference in cell apoptosis was observed whether the cells were transfected with the Control Oligos, the let-7a precursor or the let-7a inhibitor (data not shown), confirming the results from a previous study which showed that under control conditions, let-7a does not affect apoptosis [31].

STAT3 is a direct cellular target of let-7

To elucidate the cellular targets through which let-7 affects cell proliferation, we performed *in silico* analysis with different miRNA target prediction algorithms to identify putative let-7 targets. Both miRanda [32,33] and PicTar [34] predicted STAT3, the central mediator of JAK/STAT pathway, playing a role in cell proliferation, as a putative let-7 target. Several studies have previously demonstrated the role of HBx in activating STAT3 [35,36]. Hence, we proceeded to investigate whether HBx can also regulate STAT3 expression through down-regulation of let-7 miRNAs. One strong putative let-7 binding site was identified by miRanda along STAT3 3′UTR (http://www.microrna.org/microrna/home.do). To validate whether let-7 directly targets STAT3, we cloned the wild-type 3′UTR of STAT3 as well as a mutant 3′UTR in which the putative let-7 binding site is mutated, downstream of the β-galactosidase (β-gal) reporter gene (Fig. 4A). As shown in Fig. 4B, introduction of let-7a into cells containing wild-type STAT3 3′UTR reporter construct, resulted in significantly lower (p <0.001) β-gal activity compared to cells carrying the mutant STAT3 3′UTR reporter construct in which the let-7 binding site is mutated. No significant difference was observed between cells carrying wild-type or mutant STAT3 3′UTR reporter construct when Control Oligos were introduced (Fig. 4B). The endogenous STAT3 transcript and protein expression were also significantly decreased (p <0.001) when let-7a precursor was introduced (Fig. 4C). Conversely, when the let-7a inhibitor was introduced, cells carrying the wild-type 3′UTR of STAT3 displayed significantly higher (p <0.01) β-gal reporter activity compared to cells carrying the 3′UTR of STAT3 in which the let-7 binding site was mutated (Fig. 4B). Similarly, the endogenous STAT3 transcript and protein expression were also significantly increased (p <0.01) when let-7a inhibitor was introduced (Fig. 4C). As a positive control, we concurrently examined whether the addition of let-7a precursor or inhibitor into cells affected c-Myc, a previously validated cellular target of let-7a [37]. As indicated in Fig. 4C (right panel), similar decrease and increase in c-myc protein expression was observed when let-7a precursor and inhibitor, respectively, were introduced into the cells. Hence, these data suggest that let-7a physically interacts with the 3′UTR of STAT3 to negatively regulate its cellular expression. To ascertain that STAT3 is indeed a target through which let-7a affects cell proliferation, we knocked down STAT3 with a siRNA that specifically targets STAT3, and examined its effect on cell proliferation. As shown in Fig. 4D, knock-down of STAT3 reduces cell proliferation (rightmost lane), phenocopying the effect of let-7a over-expression (middle lane). Supplementary Fig. S5 shows the level of expression of let-7a and STAT3 when let-7a precursor and si-STAT3 were introduced into the cells. Moreover, STAT3

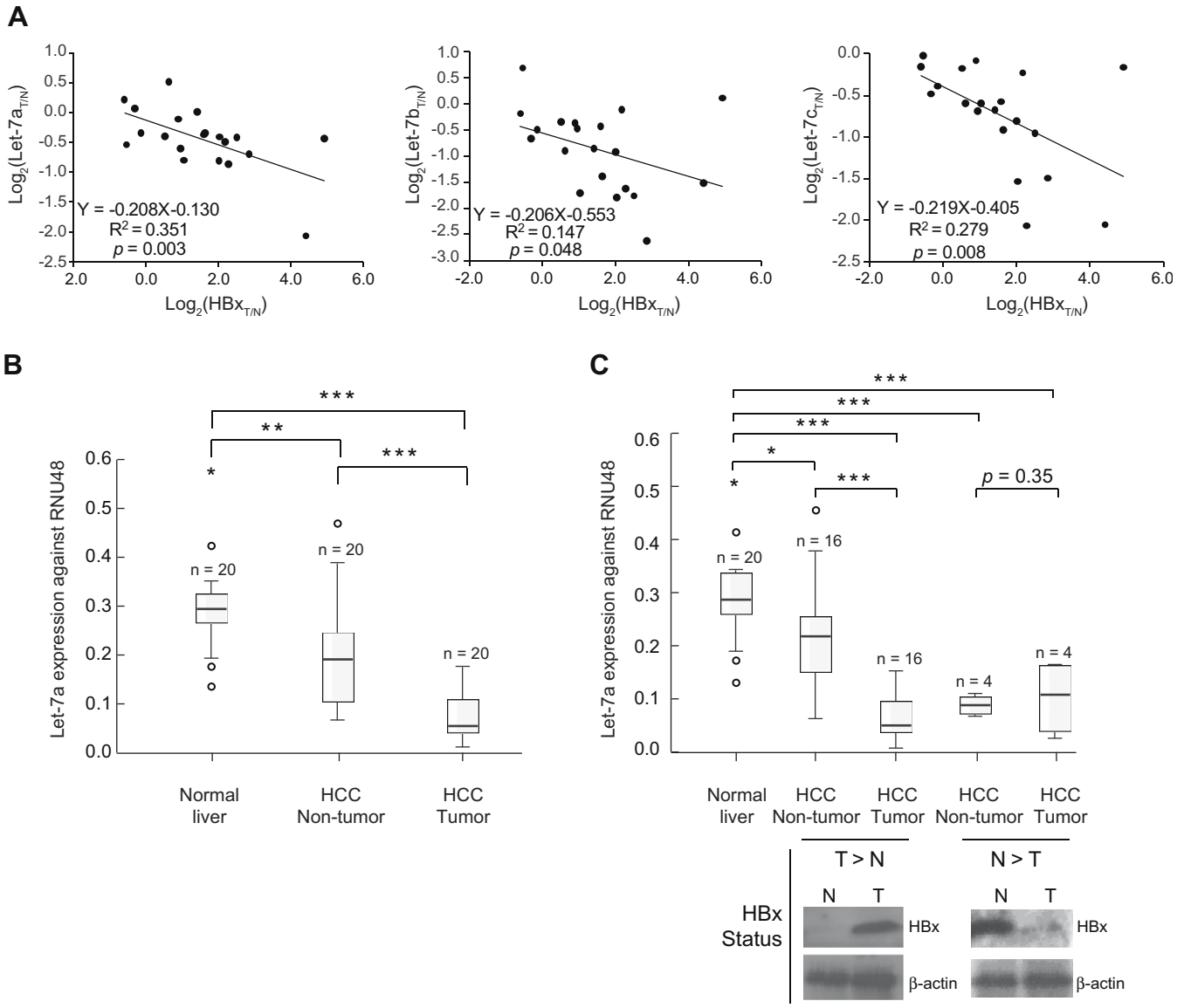

Fig. 2. HBx expression is inversely correlated with let-7a, let-7b, and let-7c expression in HCC patients. (A) Scatter plots showing the statistically significant correlation in the relative expression of HBx and let-7a (p <0.01), let-7b (p <0.05), and let-7c (p <0.01) in the tumor versus paired adjacent non-tumorous tissues of 20 HCC patients. Each spot represents data from one HCC patient presented in the $\log_2$ scale, and the linear regression line is represented by the solid line. (B) Let-7a expression is lower in HCC patient samples than in normal liver samples. Box plot of let-7a expression in the normal liver samples from 20 metastatic colorectal cancer patients (normal liver), the adjacent non-tumorous liver (HCC non-tumor), and tumor tissues (HCC-tumor) from 20 HBV-associated HCC patients. (C) Let-7a expression is only significantly reduced in HCC-tumors where HBx is selectively over-expressed, compared to the paired adjacent non-tumor tissues. Box plot of let-7a expression in 20 normal liver samples (Normal Liver), 16 HCC patients with higher HBx in tumor compared to the paired non-tumorous tissues, and 4 HCC patients with lower HBx in tumor than their paired non-tumorous tissues. Representative Western blots of HBx versus B-actin are shown below the box plot to illustrate the HBx status between the two groups of HCC patients. *p <0.05, **p <0.01 and ***p <0.001.

expression is also found to positively correlate with HBx expression in HCC patients (Supplementary Fig. S6). Taken together, our data demonstrate that let-7a regulates cell proliferation at least partially through its direct target, STAT3.

Down-regulation of let-7 by HBx supports cell proliferation in HBx-expressing cells

To reaffirm the relationship between let-7a and STAT3 in HBx-expressing cells, we examined the expression of let-7a and STAT3 in HepG2 cells transduced with Control or HBx recombinant adenoviruses respectively. As evident in Fig. 5A (left panel), let-7a shows significantly reduced expression (p <0.05) while STAT3 expression is significantly increased at both the transcript (p <0.01) and protein (p <0.05) level, in HBx-expressing cells versus control cells. As HBx down-regulated let-7, and let-7 negatively regulates cell proliferation, we hypothesized that HBx up-regulates cell proliferation through let-7a. Interestingly, cell proliferation is not significantly different in control- versus HBx-infected cells (Fig. 5B, leftmost panel, 1st two bars).

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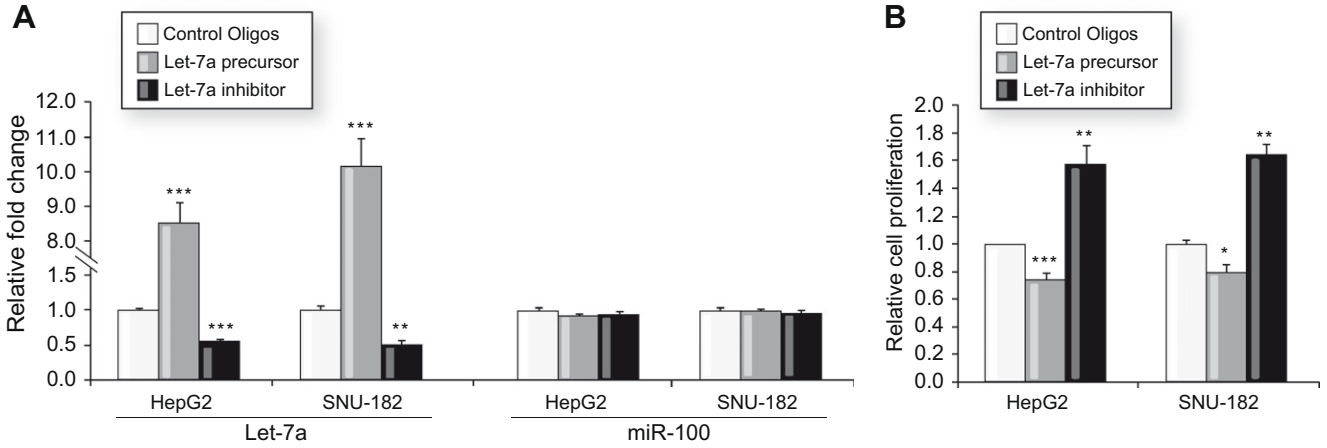

Fig. 3. Let-7 negatively regulates cell proliferation. (A) Relative let-7a (left two panels) and miR-100 (right two panels) expression normalized against RNU48, measured using reverse-transcription Taqman real-time PCR, in HepG2 and SNU-182 cells transfected with Control oligos, let-7a precursor or let-7a inhibitor. (B) Relative cell proliferation measured using the WST-1 assay in the same experimental setup. Data presented as mean ± SE from three independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001.

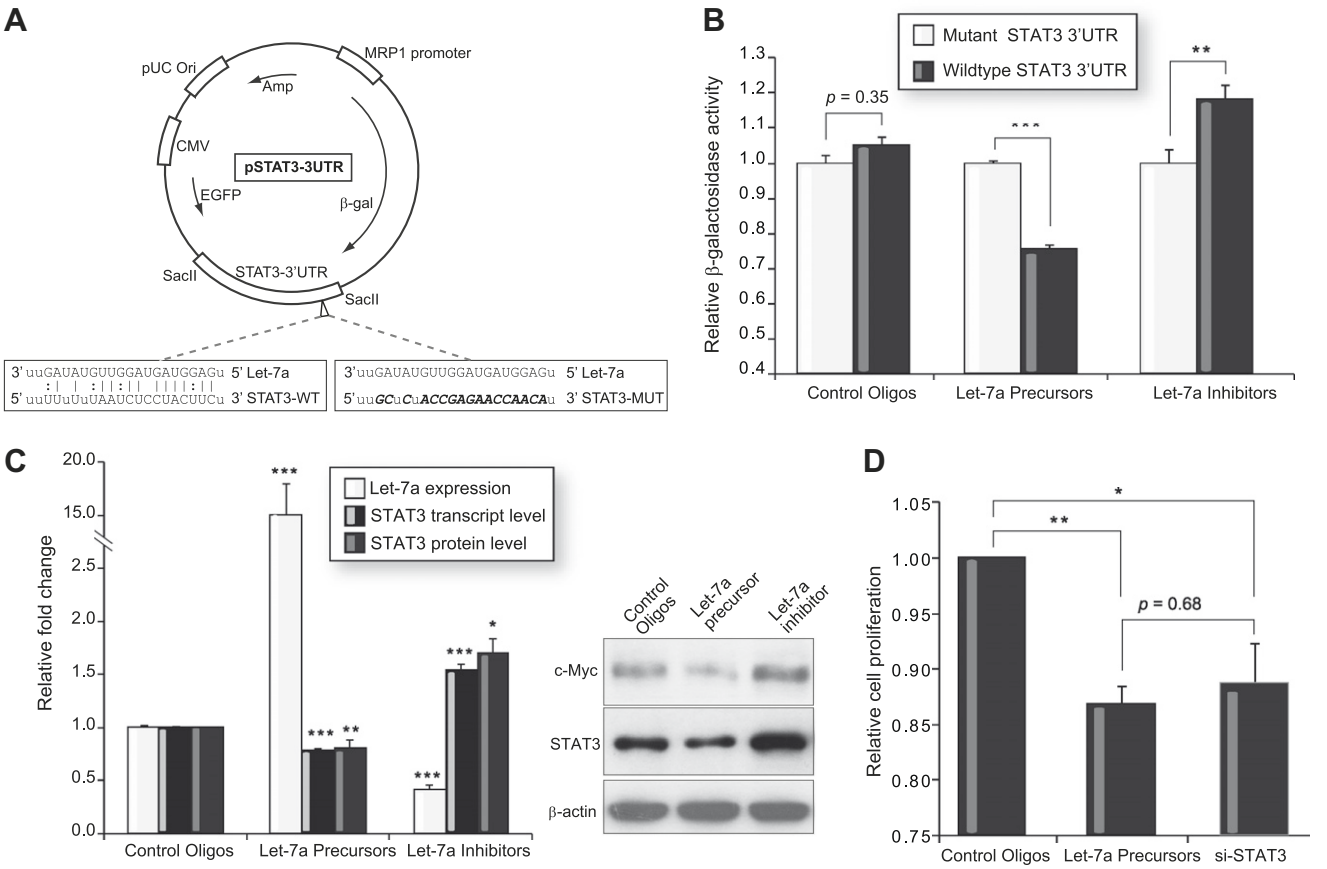

Fig. 4. Characterization of STAT3 as a direct cellular target of let-7. (A) Schematic diagram of the constructs utilized to validate that the let-7 targets the 3′UTR region of STAT3. The wild-type STAT3 3′UTR carrying the predicted let-7 binding site (left box) or the mutant STAT3 3′UTR carrying the mutated let-7 binding site (right box with the mutation residues in bold) were cloned downstream the β-galactosidase reporter gene driven by MRP1 promoter. (B) Effect of let-7a on STAT3 3′UTR examined through normalized β-galactosidase activity in cells co-transfected with Control oligos, let-7a precursor or let-7a inhibitor and wild-type STAT3 3′UTR (black bar) or mutant STAT3 3′UTR reporter construct (white bar). (C) Left panel: Graph depicting relative fold change of let-7a expression (normalized against RNU48) (white bar), STAT3 transcript (grey bar) and protein (black bar) expression (normalized against β-actin) in HepG2 cells transfected with Control oligos, let-7a precursor or let-7a inhibitor. Right panel: Representative Western blot showing protein expression of c-Myc, and STAT3 against β-actin in cells transfected with Control oligos, let-7a precursor or let-7a inhibitor. (D) Cells whose STAT3 expression is inhibited show similar proliferation phenotype as cells over-expressing let-7a. Relative cell proliferation of HepG2 cells transfected with Control oligos, let-7a precursor or siRNA against STAT3 measured using the WST-1 assay. Expression of let-7a and STAT3 after the introduction of either let-7a or siRNA against STAT3 can be found in Supplementary Fig. S5. Data presented in Fig. 4B and D are expressed as mean ± SE from three independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001.

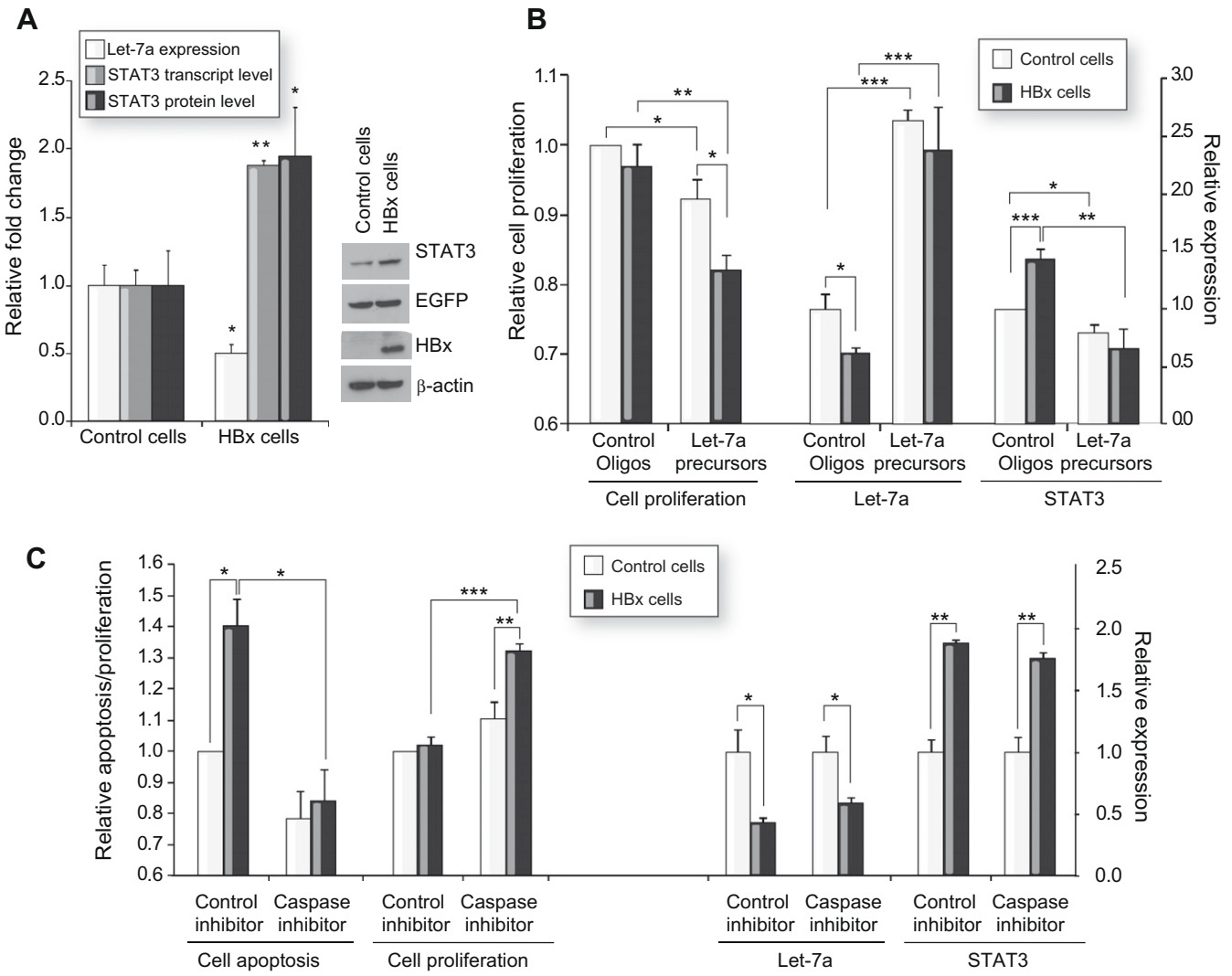

Fig. 5. HBx-expressing cells show increased cell proliferation upon treatment with apoptosis inhibitor. (A) Left panel: Relative expression of let-7a (white bar), STAT3 transcript (grey bar), and protein (black bar) in HBx-expressing HepG2 cells versus control cells. Right panel: Representative Western blotting showing protein expression of STAT3, EGFP, and HBx against β-actin. (B) Relative cell proliferation measured with WST-1 assay (leftmost panel) and relative expression of let-7a and STAT3 (right two panels) in HBx or control cells transfected with either Control Oligos or Let-7a precursors. (C) Left two panels: Relative cell apoptosis measured with Annexin V assay and relative cell proliferation measured with WST-1 assay in HBx or control cells treated with control or apoptosis inhibitor, zVAD. Right two panels: Corresponding let-7a and STAT3 expression under the same experimental conditions. Data presented are expressed as mean ± SE from three independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001.

Nonetheless, when let-7a precursors were introduced, both let-7a and STAT3 expression in the HBx cells was restored to a similar level than in control cells (Fig. 5B, middle and rightmost panels). Introduction of let-7a also results in the significant inhibition of cell proliferation in both control- and HBx-expressing cells (Fig. 5B, leftmost panel), with the reduction of cell proliferation in HBx-expressing cells being twice as much as that observed in control cells (p <0.05). As HBx has long been known to be a pleiotropic protein implicated in both cell death [18] and cell proliferation [38], our data indirectly suggest that HBx's effect on cell apoptosis may negate its effect on cell proliferation.

To reaffirm that HBx's role in cell proliferation is through its down-regulation of let-7a, we limited the effect of HBx on apoptosis by treating the cells with a general apoptosis inhibitor, zVAD, before assaying for cell proliferation. As shown in Fig. 5C, when apoptosis was not inhibited, HBx-expressing cells showed similar cell proliferation despite ~50% more cell death, compared to the control cells. HBx-expressing cells only showed significantly higher cell proliferation (~30%, p <0.01) than the control cells upon treatment with apoptosis inhibitor. The treatment with control or apoptosis inhibitor did not affect HBx's ability to down-regulate let-7a and up-regulate STAT3 (Fig. 5C). The above observations thus provide direct evidence that the pleiotropic HBx can influence both cell death and cell proliferation, and HBx-mediated down-regulation of let-7 may function to support cell proliferation in HBx cells. Taken together, these data further confirm the pleiotropic nature of HBx protein and demonstrate that HBx-mediated down-regulation of let-7 and up-regulation of STAT3 play a role in maintaining cell proliferation in order to counter-balance its effect on cell apoptosis.

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Discussion

The HBx protein plays an important role in hepatocellular carcinogenesis. Our current understanding of the role of HBx focuses on its ability to deregulate cellular gene expression at the transcription level through its interaction with many cellular factors [6]. In this study, we provided evidence that HBx can also deregulate cellular gene expression at the post-transcriptional level through the deregulation of cellular miRNAs. It is not uncommon for viruses to develop mechanisms to suppress RNA interference, which is an important antiviral defense. HIV-1 was recently reported to globally suppress host microRNA expression [39]. The Epstein-Barr virus latent membrane protein 1 was reported to activate miRNA-155 transcription [40]. This is the first study which demonstrates that the hepatitis B viral protein, HBx, also plays a role in deregulating cellular miRNAs.

Of the 286 human miRNAs that were examined in this study, HBx was found to significantly up-regulate the expression of 7 miRNAs and down-regulate the expression of 11 cellular miRNAs respectively (Fig. 1A and B). Many of these HBx-deregulated miRNAs were also reported to be similarly deregulated in many human diseases, and having functional impact on important cellular pathways such as cell death and cell proliferation. Of the seven up-regulated miRNAs, miR-125a is also up-regulated in human thyroid papillary carcinoma [41] and type II diabetes in a mouse model [42], and targets the tumor suppressor TP53 [43]. miR-199a was reported to be over-expressed in tumors of human ovarian cancer [44], and to regulate NF-κB activity by targeting IKKβ [45]. HBx has been previously reported to play a role in TP53 [46] and NK-κB pathways [47]. Our current data suggest that HBx may also regulate these important cellular pathways through the modulation of cellular miRNAs. Of the other HBx up-regulated miRNAs, miR-99b, miR-191, and miR-342 are also known to be up-regulated in myeloma [48], colorectal cancer [49] and breast cancer [50], respectively, and miR-30c is required for vertebrate hepatobiliary development [51]. However, further studies are needed to understand the role of HBx in up-regulating these miRNAs in the liver context. The 11 miRNAs that are down-regulated by HBx belong to three families of miRNAs, implicated in cell proliferation or apoptosis. miR-20a and miR-106a belong to the miR-17 family which is comprised of six members (miR-17, miR-20a/b, miR-106a/b, and miR-93) [52]. Five members of this miR-17 family, with the exception of miR-20b, were examined in our microarray and they were all generally down-regulated (data not shown). miR-20a is located in the well-known oncogenic miR-17–92 cluster that is over-expressed in many forms of cancer [27], and the repression of members of this cluster such as miR-20a leads to increased apoptosis [53]. The down-regulation of miR-106a predicted shortened disease free survival in human colon cancer patients [54]. miR-196a was reported to regulate cell proliferation and osteogenic differentiation in human adipose tissue-derived mesenchymal stem cells [55]. Significantly, all nine mature sequences of human let-7 family of miRNAs were validated to be down-regulated by HBx in human liver HepG2 cells. Let-7 miRNAs are important regulators of development, differentiation, and cell proliferation. Their down-regulation was frequently observed in many cancer types including HCC [27]. The global pattern of HBx-deregulated miRNAs can potentially target many cellular genes and have an impact on a number of cellular processes such as cell proliferation and apoptosis, partly explaining the pleiotropic nature of HBx protein.

As HBx is frequently over-expressed in the tumors of HBV-associated HCC, HBx-mediated down-regulation of let-7 may be one of the mechanisms responsible for the reduced presence of let-7 in HCC [56]. This hypothesis was supported by the significant inverse correlation between HBx and let-7a, let-7b, and let-7c expression in HCC patients. We further examined let-7a expression in 20 normal liver samples from colorectal patients who underwent surgery to remove their liver metastasis. We found that let-7a expression was significantly lower in both tumor and non-tumorous liver tissues from HCC patients where HBx expression is evident, compared to the normal liver tissues from colorectal cancer patients (Fig. 2B). Moreover let-7a expression was significantly lower in tumor compared to the adjacent non-tumorous tissues in HBV^+ HCC patients and its expression was inversely correlated with the status of the HBx protein in the paired tumor versus non-tumorous tissues (Fig. 2C). Taken together, our data demonstrate the clinical relevance of the observed HBx-mediated down-regulation of let-7 in the cell-line studied. Due to practical difficulties in obtaining samples from non-HBV-associated HCC patients, the role of let-7 in non-HBV-associated HCC remains to be investigated. The exact mechanism through which HBx down-regulates let-7 also remains unclear. We hypothesize that HBx can either bind specific cellular transcription factors to repress let-7 transcription or interact with specific cellular factors to interfere with let-7 maturation. Lin28 was recently identified to negatively regulate let-7 maturation [57], and it would be interesting to explore if HBx acts through Lin28 to negatively regulate let-7 maturation.

Let-7, the first miRNA identified in humans, was reported to target a number of important cellular oncogenes such as MYC [37], RAS [58], and HMGA2 [59]. In this study, we identified and experimentally validated another direct cellular target of let-7: STAT3, which is an important member of the JAK/STAT pathway. STAT3 was reported to be involved in many cellular processes including cell growth, survival, metastasis, angiogenesis, and immune suppression, all of which favors tumor formation and progression [60]. STAT3 was also known to play critical role in liver regeneration, and over-activation of STAT3 may play a role in contributing to HCC [61,62]. In fact, constitutive activation of STAT3 alone is sufficient to transform normal mouse fibroblasts [63]. Hence, STAT3 expression and activity are highly regulated via different mechanisms at different levels under physiological conditions. Mechanisms of STAT3 inhibition include inhibition of Jak kinase activity through the activation of SOCS1 and SOCS3, also known as suppressors of cytokine signaling proteins [64] and protein inhibitors of activated STAT3 (PIAS) [65], or through the modulation of specific phosphatases such as protein phosphatase 2A (PP2A) [66]. The finding that let-7 microRNAs target STAT3, reveals an additional level of regulation of STAT3 expression, through modulation of its transcript/protein abundance, carried out by microRNAs. miRanda predicted a total of 122 putative miRNA binding sites along the 3′UTR of STAT3 (data not shown) suggesting that the STAT3 may be highly regulated not only at several different levels mentioned above but also at the post-transcriptional/translational level by several different miRNAs including let-7. Over-activation of STAT3 is frequently observed in solid tumors including breast, ovary, and prostate tumors [60], where let-7 is also frequently under-represented [27]. However, STAT3 seems to be only one of several let-7 cellular targets through which let-7 acts to negatively regulate cell proliferation, since, completely inhibiting STAT3 alone, does not

completely inhibit cell proliferation (Suppl Fig. S5 and Fig. 4D). This is not surprising, considering that let-7 was also reported to target MYC [37] and RAS [41], both of which are important factors involved in cell proliferation. The fact that let-7 can target MYC, RAS, STAT3, and probably other proliferation factors yet to be discovered, further demonstrates the role of let-7 as a master regulator of cell proliferation, whose loss of function will result in the deregulation of crucial cellular processes, leading to tumorigenesis. Unfortunately, we could not observe a statistically significant correlation between let-7a and STAT3 expression in the 20 HCC patients we examined (data not shown). This might be due to the fact that STAT3 regulation is very complex and additional HCC patient samples may need to be examined to better stratify the samples and reveal any potential correlation.

Although the down-regulation of let-7 promoted cell proliferation (Fig. 3) and HBx was found to down-regulate let-7 expression (Figs. 1 and 5A), HBx-expressing cells do not seem to show increased cell proliferation compared to control cells under normal conditions (Fig. 5B). This is not surprising as HBx is also known to promote cell apoptosis in HepG2 cells [67]. To dissect the effect of HBx on cell proliferation from cell death, we proceeded to inhibit cell death by treating the cells with a general caspase inhibitor. When cell apoptosis is inhibited, HBx cells showed significantly higher cell proliferation than the control cells (Fig. 5C). Thus, our results strongly suggest that HBx can concurrently impact on both cell death and cell proliferation and HBx-mediated let-7 down-regulation functions to support cell proliferation in HBx cells. These data are consistent with previous reports showing that HBx is a pleiotropic viral protein involved in both cell proliferation [38] and cell death [18]. In the multi-stage, multi-hit process of tumorigenesis, HBx may function as an opportunistic factor that can potentially help promoting tumor formation partially through the down-regulation of let-7 miRNAs in cells where cell apoptosis is impaired. This could perhaps explain why different research groups observe different phenotypes in HBx-expressing cells [18,38,67] and mice [12,19], since the predominant phenotype of HBx depends on other factors that make up the microenvironment in which the function of HBx is evaluated.

In summary, this is the first report highlighting the role of the hepatitis viral protein HBx, in regulating cellular microRNAs expression. The HBx protein deregulates the expression of at least 18 cellular miRNAs, including the entire family of let-7 in HepG2 cells. Notably, a significant inverse relationship between the expression of the HBx protein and the expression of let-7a, let-7b, and let-7c was observed in HCC patients highlighting the clinical relevance of our observations. Let-7a was found to negatively regulate cell proliferation, at least in part, by targeting STAT3. HBx-mediated let-7 down-regulation supports cell proliferation in HBx-expressing cells. Hence, HBx can deregulate cellular pathways through yet another mechanism by interfering with the expression of cellular miRNAs**.** Our findings thus offer a new perspective in understanding both the pleiotropic nature of HBx viral protein and its contribution to HCC.

Conflicts of interest

The authors declared that they do not have a relationship with the manufacturers of the materials and they did not receive any funding from the manufacturers to carry out their research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.12.043.

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Role of miR-224 in hepatocellular carcinoma: a tool for possible therapeutic intervention?

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, characterized by high mortality rate and poor prognosis. Our understanding of the HCC pathology is still very much fragmented and little progress has been made to improve the clinical outcome of HCC patients. While recently discovered microRNA deregulation in HCC has added to the complexity of our understanding of HCC, it has also presented promising novel approaches to understand, diagnose and treat HCC. Here, we highlight one miRNA, miR-224, which has been more consistently reported to be upregulated in HCC than other miRNAs. We will discuss the validated and predicted functional roles of this miRNA in HCC, speculate on the possible mechanism for its upregulation in HCC and explore the potential of miR-224 as an exciting novel biomarker for the early detection of liver malignancies as well as a novel therapeutic target for HCC treatment.

KEYWORDS: biomarker ■ cancer ■ early detection ■ hepatocellular carcinoma ■ liver malignancies ■ miR-224 ■ miRNA ■ prognosis

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer related death worldwide, with an annual global mortality of more than 600,000 deaths [1,101]. The main etiological factors associated with HCC, whose importance varies according to the geographical locations, include chronic hepatitis viral infection (hepatitis B or C virus), long-term exposure to aflatoxin B1, excessive alcohol consumption and obesity [101]. Despite years of extensive research, HCC is still poorly understood and very little progress has been made to improve the diagnosis and prognosis of HCC. Surgical resection and liver transplantation remain the only curative treatment modalities that consistently convey survival advantage to less than 10% of HCC patients, while conventional radiotherapy and chemotherapy are largely less successful modalities for treating HCC [2]. Recent advances in miRNA research have provided new insights in understanding HCC and presented promising novel targets for early detection and better treatment of HCC.

Since the discovery of the first miRNA in *Caenorhabditis elegans* by Ambros and colleagues in 1993 [3], this class of small noncoding RNAs have been found to be abundantly expressed in viruses [4], plants [5] and animals [6]. To date, more than 1000 human miRNAs have been annotated in miRBase (Release 16) [7–9]. They form the largest class of gene regulators at the post-transcriptional/translational level and participate widely in many cellular processes. It is thus not surprising that miRNA deregulation has been reported to be associated with many human diseases including HCC. To date, a total of 14 publications have described miRNA deregulations in HCC through systematic profiling of miRNA expression in HCC tumors versus either paired adjacent nontumor or unpaired normal liver tissues (Table 1) [10–23]. Unfortunately, as highlighted in a previous review [24], no single miRNA is consistently reported to be deregulated by all 14 studies and only a limited partial overlap exists in reported deregulated miRNAs from different studies. As shown in Table 1, the number of HCC patient samples profiled in these studies is relatively small, ranging from three to 104 patients, with ten studies having a sample size of fewer than 30 patients. As HCC is a highly heterogeneous disease with many etiologic factors, the small sample size may partially account for the inconsistencies in the various reports. In addition, as different profiling methods such as microarray, quantitative PCR, cloning and bead-based microarray were employed in these 14 studies, the specificity and sensitivity associated with each of these profiling methods and the different number of mature miRNAs examined in each study may have also resulted in a slightly different set of miRNAs reported to be differentially expressed in HCC. A coordinated international effort which employs a consistent profiling strategy on a large sample set that sufficiently stratifies HCC patients based on etiology and ethnicity would be needed to provide a more coherent overview of miRNA deregulation in HCC.

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Table 1 . Recent publications on systematic profiling of miRNA deregulation in human hepatocellular carcinoma.

Study	Year	Profiling method	Sample size	Mature miRNAs examined (n)	Differential miRNAs identified (n)	Overexpressed				Underexpressed		Ref.
						miR-21	miR-221	miR-222	miR-224	miR-122	miR-199a	
Murakami *et al.*	2006	Microarray	25	180	6	-	-	-	↑	-	↓	[10]
Gramantieri *et al.*	2007	Microarray	17	238	28	-	↑	-	-	↓	↓	[11]
Meng *et al.*	2007	Microarray	3	-	15	↑	↑	↑	-	↓	↓	[12]
Huang *et al.*	2008	Microarray	10	331	33	↑	↑	-	-	-	-	[13]
Wang *et al.*	2008	qPCR	19	182	20	↑	↑	↑	↑	-	-	[14]
Jiang *et al.*	2008	qPCR	54	196	15	↑	↑	-	-	-	↓	[15]
Varnholt *et al.*	2008	qPCR	52	80	27	-	-	-	-	-	-	[16]
Ladeiro *et al.*	2008	qPCR	55	250	6	↑	-	↑	↑	↓	-	[17]
Connolly *et al.*	2008	MiRNA cloning	19	-	18	↑	-	-	-	↓	-	[18]
Su *et al.*	2009	Microarray	20	308	28	-	-	↑	↑	-	↓	[19]
Huang *et al.*	2009	Bead-based flow	20	114	30	-	↑	↑	↑	↓	↓	[20]
Ura *et al.*	2009	qPCR	26	188	29	↑	↑	↑	-	↓	↓	[21]
Pineau *et al.*	2010	Microarray	104	215	12	↑	↑	↑	↑	-	-	[22]
Wong *et al.*	2010	qPCR	20	156	36	↑	-	↑	↑	-	↓	[23]

Sample size refers to the number of HCC patients included in the miRNA profiling experiment. Number of differential miRNAs include mature miRNA sequences and exclude miRNA (the minor strand from the opposite arm of the precursor miRNA) sequences.*
↑: miRNAs overexpressed in HCC tumors; ↓: miRNAs underexpressed in HCC tumors; -: miRNA of interest is not profiled or not found to be significantly differentially expressed.
HCC: Human hepatocellular carcinoma.

miRNAs which have been more consistently reported to be aberrantly expressed in multiple studies, irrespective of the nature of the patient samples or the profiling technologies, are most likely to be involved in the general mechanism of hepatocellular carcinogenesis. Table 1 summarizes the four upregulated miRNAs (miR-21, miR-221, miR-222 and miR-224) and two downregulated miRNAs (miR-122 and miR-199a) which have been consistently reported by at least six out of the 14 profiling studies. Indeed, these commonly deregulated miRNAs target crucial cancer-implicated pathways. miR-21 is the most commonly upregulated miRNA in both solid and hematological tumors [25], and inhibits cell apoptosis by targeting phosphatase and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4) [26]. miR-221/222 promote cell proliferation by targeting p27/CDKN1B to stimulate G1-S transition [27] and inhibits cell apoptosis by targeting PTEN [28]. miR-122 is the most abundant liver miRNA involved in liver homeostasis [29], and the loss of miR-122 in HCC correlates with suppression of hepatic phenotype and gain of metastatic properties [30].

In this article, we will discuss the functional impact of miR-224 upregulation in HCC, and speculate on the possible mechanism for its upregulation and its potential role as a promising therapeutic and diagnostic target for HCC.

miR-224 upregulation impacts multiple cellular pathways

Each miRNA can potentially regulate hundreds of cellular targets and affect multiple cellular pathways. One way to gain insights into the functional role of miR-224 is to examine the cellular genes which miR-224 targets. As most of miR-224 direct cellular targets are unknown, *in silico* miRNA target prediction algorithms are the second best available option. miRecords, which is an online resource that compiles predicted miRNA targets produced by 11 established miRNA target prediction programs, was used to gain some insights into the putative targets that miR-224 potentially regulate. A total of six of these 11 prediction programs, miRanda, mirTarget2, miTarget, PITA, RNAhybrid and TargetScan, predict targets for human miR-224 [31]. A total of 52 putative miR-224 targets, mapping into 39 gene ontology (GO) annotated functional processes, were predicted by at least five prediction programs (Table 2). These *in silico* predictions suggest that miR-224 can potentially affect many cellular processes associated with cancer, including transcription, cell differentiation, cell death, growth and cell proliferation. Interestingly, miR-224 is predicted to affect both cell death and cell growth (Table 2). This seemingly opposing role of miR-224 was also

observed experimentally where miR-224 was found to increase both cell proliferation and apoptotic cell death [14,32]. One of the predicted miR-224 targets in cell death process, apoptosis inhibitor 5 (API5) (Table 2), has also been validated to be a direct cellular target through which miR-224 sensitizes cells to apoptotic cell death in liver cells. The target through which miR-224 influences cell proliferation remains unknown. The *in silico* prediction program suggests four nonredundant genes are involved in processes of cell growth and cell proliferation (Table 2). Of these four genes that are potential targets of miR-224, SMAD family member 4 (SMAD4) is the only putative miR-224 target gene that is consistently highlighted. SMAD4, which is frequently dysregulated in cancer, is the central mediator for transforming growth factor β (TGF-β) pathway and negatively regulates cell proliferation. Hypothetically, the possible downregulation of SMAD4 by miR-224 overexpression will derepress the TGF-β mediated inhibition on cell growth, resulting increased cell proliferation. This hypothesis is supported when Yao *et al.* reported that miR-224 is regulated by TGF-β/SMAD pathway and targets SMAD4 in mouse granulosa cells [33]. If this is true in human liver cells, it suggests the physiological role of miR-224 in the negative feedback loop that controls TGF-β signaling. miR-224 overexpression in HCC will significantly deregulate the TGF-β signalling pathway and facilitate unregulated proliferation.

The role of miR-224 in increasing both cell proliferation and cell death may initially seem contradictory. However, similar to miR-224, many other oncogenes including *MYC* and *E1A* were found to sensitize cells to apoptosis upon minor insults that normal cells can usually resist [34,35]. Like these oncogenes, the dual role of miR-224 to influence both cell proliferation and apoptosis may provide the selective pressure for cells to override apoptosis during the multistage process of tumorigenesis, resulting in a resistant population of proliferating cells which may eventually accumulate enough genetic mutations for transformation to occur.

miR-224 may also play an important role in metabolic processes and this is highlighted by the fact that 28 out of the 52 putative miR-224 targets are enriched in metabolic processes (Table 2). The Warburg effect of aerobic glycolysis is a key metabolic hallmark of cancer [36] and the activation of many oncogenes, such as *RAS* [37] and *MYC* [38], has been shown to drive changes in cell metabolism. Since *in silico* prediction has provided some useful clues in understanding the functional roles of miR-224 in the aspect of cell proliferation and cell apoptosis, it will be very exciting to examine miR-224's involvement in cancer metabolism, using a similar strategy. In addition, miR-224 overexpression is also recently found to increase cell migration, invasion [33] and anchorage independent cell growth [39]. Taken together, these data suggest that miR-224 could be a potent oncogenic miRNA targeting multiple cancer-implicated pathways and the upregulation of miR-224 in HCC seems to favor cellular transformation. It may be worthwhile to evaluate whether miR-224 status correlates with clinical outcome for HCC patients.

Possible mechanisms for miR-224 upregulation in HCC

Like its gene counterpart, the regulation of miRNA expression is very complex. miRNA expression can be regulated at multiple levels including DNA amplification/deletion, epigenetic modification, transcription, miRNA processing and decay. The expression of specific miRNAs such as let-7 has also been shown to associated with specific etiological factors such as hepatitis B virus infection [40].

Although the mechanism for the upregulation of miR-224 in HCC has yet to be elucidated, the genomic location of this miRNA may provide some clues about its regulation. As shown by Figure 1, miR-224 resides in the same cluster as miR-452 in the intron 6 of γ-aminobutyric acid receptor subunit epsilon (*GABRE*) gene, located near the end of q arm of chromosome X, in ChrXq28, a region known to be transcriptionally quiescent. As the incidence of HCC is more than twice as high in males than in females [101], the X-linked nature of miR-224 upregulation may partly explain this sex bias in HCC.

Although the ChrXq28 locus has been reported to be duplicated in a rare genetic disease affecting sexual development [41], it is unlikely to be the explanation for the upregulation of miR-224 expression in HCC since the duplication of ChrXq28 is rare and causes severe phenotypes such as mental retardation and death before the age of 25 years [42]. Nonetheless, ChrXq28 is one of the best characterized human genomic regions hosting many cancer/testis (CT) antigen genes, a class of immunogenic proteins which are physiologically expressed only in the male germ cells but not in somatic cells. In many cancers (e.g., melanoma,

Table 2. Gene ontology term enrichment of 52 putative miR-224 targets predicted by at least five prediction algorithms.

GO term	GO ID	Genes annotated to the GO term
Metabolic process†	0008152	*ACSL4, AFF3, ATF2, BRPF3, BTRC, CASC3, DPYSL2, HMGCR, MAPK14, MED13L, NCOA6, NUAK1, POLR1D, POU3F2, PPAP2B, PRPF4B, RAD51L1, RUNX2, SDC4, SMAD4, SR140, TCERG1, TRIM9, UBE2D3, WTAP, YOD1, ZNF207, ZNF423*
Primary metabolic process†	0044238	*ACSL4, AFF3, ATF2, BRPF3, BTRC, CASC3, DPYSL2, HMGCR, MAPK14, MED13L, NCOA6, NUAK1, POLR1D, POU3F2, PPAP2B, PRPF4B, RAD51L1, RUNX2, SMAD4, SR140, TCERG1, TRIM9, UBE2D3, WTAP, YOD1, ZNF207, ZNF423*
Regulation of biological process	0050789	*AFF3, AMIGO2, API5, ATF2, BTRC, CASC3, DPYSL2, ITM2B, MAP1B, MAPK14, MED13L, NCOA6, POU3F2, PPAP2B, RAB10, RUNX2, SDC4, SMAD4, TCERG1, UBE2D3, ZNF207, ZNF423*
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0006139	*AFF3, ATF2, CASC3, DPYSL2, HMGCR, MED13L, NCOA6, POLR1D, POU3F2, PPAP2B, PRPF4B, RAD51L1, RUNX2, SMAD4, SR140, TCERG1, UBE2D3, WTAP, ZNF207, ZNF423*
Multicellular organismal development	0007275	*ACSL4, AFF3, BTRC, DPYSL2, GGNBP2, H3F3B, HMGCR, ITM2B, MAP1B, NCOA6, POU3F2, PPAP2B, RUNX2, SDC4, SMAD4, ZNF423*
Biosynthetic process	0009058	*AFF3, ATF2, CASC3, HMGCR, MED13L, NCOA6, POLR1D, POU3F2, PPAP2B, RUNX2, SMAD4, TCERG1, ZNF207, ZNF423*
Transcription	0006350	*AFF3, ATF2, MED13L, NCOA6, POLR1D, POU3F2, PPAP2B, RUNX2, SMAD4, TCERG1, ZNF207, ZNF423*
Protein metabolic process	0019538	*BRPF3, BTRC, CASC3, MAPK14, NUAK1, PPAP2B, PRPF4B, SMAD4, TRIM9, UBE2D3, YOD1*
Cellular component organization	0016043	*ACSL4, BRPF3, DIAPH3, H3F3B, MAP1B, NCOA6, POU3F2, SDC4, SMAD4*
Cell differentiation	0030154	*ACSL4, DPYSL2, GGNBP2, MAP1B, NCOA6, POU3F2, RUNX2, SMAD4, ZNF423*
Protein modification process	0006464	*BRPF3, BTRC, MAPK14, NUAK1, PPAP2B, PRPF4B, SMAD4, UBE2D3, YOD1*
Response to stress	0006950	*CASC3, MAP1B, MAPK14, NCOA6, RAD51L1, SMAD4, UBE2D3, YOD1*
Catabolic process	0009056	*BTRC, CASC3, SMAD4, TRIM9, UBE2D3, YOD1*
Death‡	0016265	*AMIGO2, API5, ITM2B, MAPK14, UBE2D3*
Anatomical structure morphogenesis	0009653	*BTRC, MAP1B, POU3F2, PPAP2B, SMAD4*
Signal transduction	0007165	*BTRC, DPYSL2, MAPK14, RAB10, SMAD4*
Cell communication	0007154	*BTRC, DPYSL2, MAP1B, POU3F2, SMAD4*
Embryo development	0009790	*ACSL4, MAP1B, NCOA6, PPAP2B, SMAD4*
Cell death‡	0008219	*AMIGO2, API5, ITM2B, MAPK14, UBE2D3*
Organelle organization	0006996	*BRPF3, DIAPH3, H3F3B, MAP1B, SDC4*
Reproduction	0000003	*ACSL4, GGNBP2, HMGCR, PPAP2B*
Response to endogenous stimulus	0009719	*DPYSL2, H3F3B, MAP1B, NCOA6*
DNA metabolic process	0006259	*NCOA6, RAD51L1, UBE2D3*
Protein transport	0015031	*GGA3, RAB10, SMAD4*
Response to external stimulus	0009605	*ACSL4, MAP1B, MAPK14*
Cytoskeleton organization	0007010	*DIAPH3, MAP1B, SDC4*
Lipid metabolic process	0006629	*ACSL4, HMGCR, PPAP2B*
Cell proliferation§	0008283	*BTRC, POU3F2, SMAD4*
Growth§	0040007	*MAP1B, POU3F2, SMAD4*
Cell cycle	0007049	*BTRC, RAD51L1, WTAP*
Behavior	0007610	*ACSL4, MAPK14*
Cell growth§	0016049	*MAP1B, SMAD4*

These 52 miR-224 putative targets were mapped in 39 functional processes.
†*Metabolic processes.*
‡*Processes involved in cell death.*
§*Processes involved in cell proliferation.*
GO: Gene ontology.
Data taken from [102].

Table 2. Gene ontology term enrichment of 52 putative miR-224 targets predicted by at least five prediction algorithms (cont.).

GO term	GO ID	Genes annotated to the GO term
Cell–cell signaling	0007267	*DPYSL2*, *MAP1B*
Ion transport	0006811	KCTD12, SLC4A4
Response to abiotic stimulus	0009628	*MAP1B*
Cellular amino acid and derivative metabolic process	0006519	*YOD1*
Response to biotic stimulus	0009607	*YOD1*
Translation	0006412	*CASC3*
Cellular homeostasis	0019725	*POU3F2*

These 52 miR-224 putative targets were mapped in 39 functional processes.
[†]Metabolic processes.
[‡]Processes involved in cell death.
[§]Processes involved in cell proliferation.
GO: Gene ontology.
Data taken from [102].

lung carcinoma and HCC), the somatic expression of these CT antigens is derepressed mainly through epigenetic reprogramming including DNA methylation and chromatin remodeling [43]. A total of 19 CT antigens are mapped onto ChrXq28 region and two of these, *MAGEA4* and *MAGEA5*, flank the miR-224 locus (Figure 1). As tumors often express several CT antigens simultaneously [43], it is possible that the expression of miR-224 and CT antigens residing in the same chromosome region of ChrXq28 may be coordinately upregulated through epigenetic reprogramming. Such coordinated regulation of expression was suggested by the limited expression data of miR-224 and closeby genes at ChrXq28. Similar to miR-224, *GARBE* (miR-224 host gene) and *MAGEA4* (~30 kbp away from miR-224 locus) are also found to be upregulated in HCC [44,45], suggestive of coregulation between miR-224 and neighboring genes. As CT antigens were reported to be upregulated epigenetically [43], it is thus likely that miR-224 is also upregulated epigenetically since it resides in the same chromosome region and seems to be coordinately upregulated in HCC although this remains to be experimentally validated. Nonetheless, with the availability of specific drugs targeting different epigenetic machineries such as DNA methylase inhibitor (5-aza-2′-deoxycytidine), histone deacetylase inhibitor (trichostatin A) and histone methylase inhibitor (3-deazaneplanocin A), the evaluation of the epigenetic regulation of miR-224 can be readily assessed. The fact that miR-224 may be coregulated with its host (*GABRE*)and neighboring genes (*MAGEA4* and *MAGEA5*), since they reside close to one another in the same chromosome, suggests that further characterization of this group of genes/miRNAs is warranted.

miR-224 was recently reported to be under the regulation of specific signaling pathways including the Src tyrosine kinase (Src) in mammary carcinoma cells [39], and the TGF-β pathway in mouse granulosa cells [33], highlighting the involvement of different players in the regulation of miR-224. We speculate that epigenetic reprogramming/chromatin remodeling precedes the binding of these transcription factors at the miR-224 locus to induce the expression of miR-224.

Potential therapeutic strategies to target miR-224 in HCC

As discussed earlier, miR-224 was reported to affect cell proliferation [14], apoptosis [14], cell migration, invasion [33] and anchorage independent cell growth [39], implicating this miRNA as an oncogenic miRNA capable of impacting multiple crucial cellular pathways. However, it remains unclear whether miR-224 would be a good therapeutic target for the treatment of HCC. If so, promising new therapeutic strategies are being developed and can be quickly adapted to target miR-224.

The first strategy involves the liver-specific delivery of short oligonucleotide-based miR-224 inhibitors to block the action of mature miR-224 in deregulating cellular processes. Antisense miR-224 inhibitors have been shown to effectively reverse phenotypes associated with miR-224 overexpression in *in vitro* models [14] although it remains to be evaluated if these *in vitro* findings can be reproduced in animal models and human subjects. One attractive advantage of this strategy is the possibility of site-specific delivery of such small oligo-based miR-224 inhibitors to liver cells through systemic intravenous administration. Zhu *et al.* has

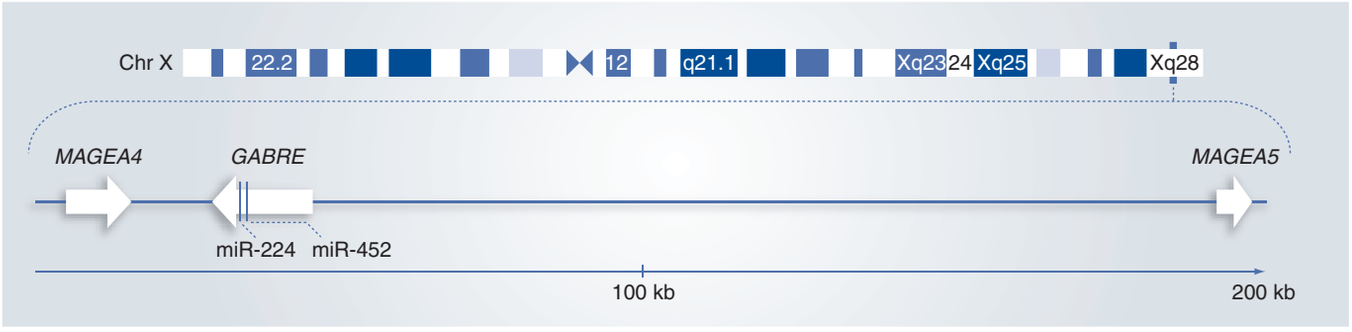

Figure 1. Genomic locus of miR-224. The top chromosomal map shows the location of miR-224 in ChrXq28 and the bottom map represents an enlargement of the 200-kb region in ChrXq28 showing the location of the miR-224/452 cluster, the host gene *GABRE* and the two flanking cancer/testis antigen genes (*MAGEA4* and *MAGEA5*).
Chr: Chromosome.

recently demonstrated the proof-of-concept for rapid site-specific accumulation of engineered oligonucleotides in liver cells through such systemic administration [46]. If proven successful, this strategy may be useful to complement liver resection as a presurgical treatment to contain and manage HCC tumors.

If the expression of miR-224 is regulated epigenetically, a second strategy would be to deliver specific drugs that interfere with the chromatin structure. Recently, targeting epigenetic machinery has emerged as an exciting new strategy for drug development, particularly for cancer and metabolic diseases. Vorinostat, a histone deacetylase inhibitor, is the first such drug approved by the US FDA in 2006 for the treatment of advanced cutaneous T-cell lymphoma [47]. However, the limitation of current epigenetic drugs, such as vorinostat, is the nonspecific nature of these drugs, targeting a large number of cellular components such as all class I and II histone deacetylases. Knowledge on the exact mechanisms of the epigenetic processes will facilitate the identification of specific compounds that targets the individual component of the epigenetic machinery. Finding a specific small molecule epigenetic inhibitor drug may prove useful not only in neutralizing miR-224 upregulation in HCC, it may also neutralize the aberrant activation of other CT antigens in ChrXq28 and other regions of the genome that are also associated with HCC. Nonetheless, as these inhibitors are general inhibitors targeting the whole chromatin structure, there is a possibility of these inhibitors silencing other essential genes. Hence, further research has to be performed to understand these drugs better.

The development of a relevant mouse model would facilitate the understanding of the oncogenic potential of miR-224 in causing HCC as well as the evaluation of the above therapeutic strategies targeting miR-224.

miR-224 may represent a useful novel biomarker for HCC or liver disease

Early detection of HCC has been difficult as the progression of HCC is largely asymptomatic. Currently, surveillance of HCC relies mainly on monitoring serum α-fetoprotein levels and regular ultrasound or computed tomography examination [48]. A definitive diagnosis of HCC depends on histological analysis via a fine needle biopsy. However, elevated α-fetoprotein as a biomarker lacks specificity and the high-cost ultrasound or computed tomography scan lacks affordability for a general screening application. A simple plasma-based test which can rapidly assess the liver status will be an important contribution to the HCC screening programs.

Although the development of miR-224 as a therapeutic target for HCC may still be a far-fetched idea, there is great potential for miR-224 to be developed as a HCC or liver disease biomarker. Under normal physiological conditions, the expression of miR-224 is very low or even undetectable [18] probably due to the transcriptional quiescent state of ChrXq28. However, as shown in Table 1, miR-224 is one of the most consistently upregulated miRNAs in HCC. Interestingly, studies which compare miR-224 expression in HCC tumors with unpaired normal liver samples [17,19] tend to report much larger (27–30-fold) changes than those that compare HCC tumors with paired adjacent nontumor samples (2.5–11-fold) [10,14], suggesting that miR-224 expression is not only upregulated in HCC, but it is also elevated in preneoplastic liver tissues which are frequently inflamed and/or cirrhotic. Murakami *et al.* found upregulation of miR-224 in chronic hepatitis and liver cirrhosis [10]. Ladeiro *et al.* reported moderate elevation (~fivefold) in miR-224 expression in benign tumors such as hepatocellular adenoma and miR-224 expression was further elevated in

tumors of HCC (>20-fold) [17]. Interestingly, the miR-224 host gene, *GABRE*, was also reported to show a similar pattern of gradual upregulation corresponding to liver disease progression [44]. Taken together, these data suggest that miR-224 expression is gradually upregulated during liver disease progression, probably due to accumulated epigenetic changes in the genomic locus, making miR-224 a useful potential biomarker to assess liver health status.

For miR-224 to be developed as a potentially useful biomarker for HCC or liver disease, it must be readily detectable in a less invasive tissue like the blood/serum/plasma. miRNAs have recently been reported to be present in bodily fluid including blood serum making miRNAs attractive novel biomarkers [49]. The hypothesis is that miRNAs from damaged tissue such as necrotic tumor tissues are released into the blood serum which will facilitate its detection through a simple plasma-based test. Of the four miRNAs (miR-21, miR-221, miR-222 and miR-224) (Table 1) that are consistently upregulated in HCC, miR-224 seems the most specific to HCC [50], although it was also found to be upregulated in thyroid tumors in one study [51]. As liver tissues are highly vascularised, miRNAs from liver damage would readily be released in the blood circulation facilitating its detection in the blood serum. Indeed, a recent study which examined the serum miRNA from 15 patients with clinically diagnosed HCC, ten with liver cirhosis and ten age-matched healthy individuals, revealed higher levels of miR-224 in the blood sera of patients with HCC (87-fold) or liver cirrhosis (14-fold) than the normal healthy patients [52]. To further develop miR-224 as a useful biomarker, a larger study with appropriate stratification according to liver conditions (e.g., healthy liver, liver hepatitis, liver cirrhosis and hepatocellular adenoma) as well as grades of HCC is warranted, so that the levels of serum miR-224 under the various liver health conditions can be determined to facilitate rapid clinical interpretation.

Conclusion

In summary, miR-224 is one of the most consistently upregulated miRNAs in HCC that potentially affects crucial cancer implicated cellular processes such as cell proliferation, apoptosis and cell migration, making it an attractive target for HCC therapy. Notably, the detection of higher levels of miR-224 in the serum of HCC patients and the observationthat miR-224 upregulation seems to correspond to different stages of liver disease progression suggests that miR-224 may represent a novel biomarker for liver disease and HCC.

Future perspective

This article summarizes the facts, inferences and speculations of miR-224 in hepatocellular carcinogenesis. To evaluate if miR-224 is indeed a useful therapeutic target for HCC, further research in both *in vitro* and *in vivo* models is needed to fully understand miR-224 and its many targets. The effective design of a therapeutic strategy targeting miR-224 would require our understanding of the exact mechanism for miR-224 upregulation. Nonetheless, miR-224 may represent a useful attractive biomarker for HCC/liver disease since it is detected at higher levels in the serum of HCC patients compared with normal individuals and its expression seems to correlate with the health status of the liver. Further research with appropriate stratification examining the serum of individuals at different stages of liver disease or HCC is required to validate this observation. We hope that this article can offer some perspective and stimulate new ideas in our quest to understand miR-224 in HCC.

Executive summary

miR-224 upregulation impacts on multiple cellular pathways

- miR-224 is reported by multiple studies as one of the more consistently upregulated miRNAs in hepatocellular carcinoma (HCC).
- miR-224 increases cell proliferation, apoptosis, migration, invasion and anchorage independent growth.

Possible mechanisms for miR-224 upregulation in HCC

- miR-224 was reported to be induced by Src and the TGF-β pathway.
- There is indirect evidence that suggests that miR-224 may be epigenetically regulated.

Potential therapeutic strategies to target miR-224 in HCC

- miR-224 upregulation in HCC can potentially be targeted through liver-specific delivery of small oligonucleotide-based inhibitors via systemic administration.
- If miR-224 is epigenetically regulated, drugs that specifically interfere with epigenetic modification may be useful.

miR-224 may represent a useful novel biomarker for HCC or liver disease

- miR-224 expression seems to correlate with the health status of the liver.
- miR-224 can be detected in the serum and its levels have been found to be higher in the serum of HCC patients when compared with normal individuals, suggesting that miR-224 has the potential to be a useful, novel, less invasive biomarker for HCC.

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